PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS

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PREFACE

In recent years, interest in the chemistry of fats and other lipids has increased remarkably. Heretofore, emphasis in biochemistry has fallen in such areas as mineral metabolism, protein chemistry, vitamins, and hormones, and by contrast the field of lipids has been somewhat neglected. With the current intensified interest in biochemical and chemical problems of the lipids, it seems logical that the progress in the field be summarized periodically.

The lipids are themselves a group of very diverse substances, grouped together through association in nature and, traditionally, through similar solubility characteristics, rather than through similarity in chemical structure. The modern methods applicable to separations, analysis, and characterization of lipid substances are likewise very diverse. Thus, the content of the Progress Series is of necessity heterogeneous. Any effort toward uniformity would defeat the purpose of the series—to bring together all available information on classes of substances and on modern techniques which may be of interest and use to lipid specialists. The chapters should be looked upon as short monographs collected in a volume for convenience.

Chapters are solicited from recognized authorities in each field, and style and content are left largely to the authors. By this means the editors hope to obtain for the reader the most authoritative information and viewpoints on a given subject. They also hope thus to preserve originality and individuality in the presentations, qualities which are often lacking in scientific writing. Thereby, for the sake of freedom, some conformity and continuity are lost.

The editors recognize a natural division of subject matter between chemistry and technology of fats. For this reason, technological advances will appear in a separate series entitled Progress in the Technology of Fats.

March, 1955 THE EDITORS

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1

PARENTERAL ADMINISTRATION OF FATS

Smith Freeman

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1

PARENTERAL ADMINISTRATION OF FATS

Smith Freeman

I. Historical Background

The need for a parenteral fluid rich in calories and of low osmotic effect has been recognized by the medical profession for many years. Leube (1895), when giving subcutaneous injections of camphor in oil to patients with cardiac failure, suggested the possible value of parenteral fat as a source of calories. Studies on the metabolic effect of intravenous fat on animals began early in the twentieth century (Mills and Murlin, 1909–10; Mills and Congdon, 1911; Koehne and Mendel, 1929). As early as 1918, emulsified fat was given intravenously to man as a vehicle for camphor or quinine (Mansfield, 1918).

The parenteral administration of milk with egg yolk or cod liver oil was among the earliest attempts to provide nourishment for patients by means of a fat-rich parenteral fluid (Whittaker, 1876; Pick, 1879). Yamakawa and associates (Yamakawa, 1920; Nomura, 1928–29; Yamakawa and Nomura, 1928; Sato, 1931) were among the first workers to attempt to provide nutrition to patients by means of an artificially prepared fat emulsion. They injected an emulsion of egg lecithin, butter and cod liver oil into several hundred patients with peptic ulcer. Somewhat later Valledor, Casas, and Gomez del Rio (1928) reported favourably on the injection of marasmic infants with fat emulsions. A more extensive study was made a few years later by Holt, Tidwell, and Scott (1935) on a group of undernourished children. Their emulsion contained egg lecithin and olive or coconut oil. The homogenized mixture could be autoclaved and had a particle size of 2 to 3 micra.

More recently studies have been made by a number of investigators regarding the metabolic and physiological effects of repeated intravenous injections of emulsified fat. From these studies, it has become apparent that although the utilization of parenteral fat does take place, numerous problems must be solved before the intravenous injection of fat becomes a safe and practical measure. Various aspects of this problem will be considered.

II. Factors Concerned in the Emulsification of Fat and Stability of the Fat Emulsion

Fat normally exists in the circulation in a highly dispersed state; even when the plasma is markedly lipaemic the largest visible globules of fat are usually 1 micron or less in diameter (Elkes, Frazer, and Stewart, 1939). These tiny

globules of neutral fat are discrete and necessarily manifest marked Brownian movement. It may be taken as a premise that a similar degree of dispersion and activity are desirable characteristics for a fat emulsion to be infused into the circulation.

The dispersion of an immiscible substance of low density into an aqueous medium entails consideration of a variety of factors which are generally recognized to influence the behaviour of colloids. These include the charge, concentration of the dispersed substance, its specific gravity, the relative density of the dispersed substance and dispersing medium, viscosity of the dispersing phase, particle size and interfacial tension. A more detailed discussion of these factors may be found elsewhere (LaVeen, Papps, and Restuccia, 1950). It is sufficient to point out here that stabilization of a fat emulsion requires that the particles be highly dispersed (1 micron or less) and stable in this state. A tendency for the globules of fat to clump, cream or coalesce, will result in an emulsion that cannot be stored or subjected to contact with surfaces without its breaking down. One must not only consider the emulsion in its original form but also its reaction to heat and contact with the various constituents contained in the circulation. The emulsion must be stable in the presence of electrolytes approximating the concentration of a 0·85 per cent solution of sodium chloride. The relatively high interfacial tension that characterizes fat emulsions in water makes it unlikely that one will ever be able to prepare a sufficiently stable emulsion by purely mechanical means. Satisfactory stability has only been possible by lowering the interfacial tension by means of surface active agents. Such compounds by their very nature tend to cause the liberation of haemoglobin from the red blood cell in the circulation. The relation of surface tension to haemolysis imposes a serious restriction on the means one may employ to meet the requirements indicated above. The dispersion of fat must minimize changes in surface activity or the surface active agents must be sufficiently labile in the body to produce no toxic effects before or after decomposition.

A great variety of emulsifying agents have been employed in preparing fat emulsions and only a few examples will be mentioned. Lecithin and related phosphatides have been used most frequently. Usually 1 to 2 per cent lecithin is used in a 10 per cent emulsion of fat. Egg yolk and soya beans have been the two most common sources of phosphatides for this purpose; crude preparations may produce granulomatous lesions. Purification and standardization of the least toxic fraction of soya phosphatides have been described by Geyer (Geyer, Mann, Young, Kinney, and Stare, 1948) who prepared a fraction with good emulsifying properties and little tendency to produce granulomatous lesions in rats. However, phosphatides are generally acknowledged to be a relatively unstable group of compounds because of the unsaturated fatty acids which they contain and it has also been demonstrated that spontaneous hydrolysis with the formation of lysolecithin may occur upon standing in solution (Frost, 1953).

The effect of proteins on the stability of fat emulsions has also received attention. It was found (LaVeen, Papps, and Restuccia, 1950) that purified

serum albumin and gelatin tended to stabilize fat emulsions while the globulin fraction of serum proteins caused fat globules to coalesce. SHAFIROFF and FRANK (1947) have described the preparation of stable fat emulsions employing gelatin as the emulsifying agent. This agent has the advantage of permitting storage of the emulsion in the gel form which reduces the tendency for creaming that may occur when liquid preparations are allowed to stand for some time.

Other agents that have been employed to emulsify fat for parenteral use include fatty acid esters of various polyhydric alcohols or sugars, bile salts, polyglycerol esters of fatty acids, cerebrosides, substituted amines, organic esters of phosphoric acid and a variety of other surface active agents too numerous to mention. The mechanical means of fat dispersion have included high pressure extrusion of the mixture through a fine orifice and the use of supersonic vibrations.* By means of surface active agents and mechanical devices fine stable fat emulsions have been produced, but it cannot be said that the products thus far prepared are without toxic effects on the animal organism when infused intravenously in considerable amounts.

III. Toxic Reactions to Infused Fat Emulsions

The infusion of relatively stable, highly dispersed emulsions of fat into human subjects or animals has been associated with a wide variety of effects, some of which are apparent immediately while other changes only become apparent when injections are given repeatedly. The rate of infusion, dispersing agents, purity of the starting materials, stability and particle size of the emulsion, as well as the characteristics of the recipient are all recognized as factors that will influence the nature and extent of the immediate reaction to fat infusion. Headache, dizziness, fluctuations in blood pressure, syncope, chills, fever, nausea, vomiting, precordial distress or tightness across the chest, an ache in the lumbar region of the spine, cough and urticaria are all symptoms that may arise from the injection of colloidal substances intravenously. Many of the symptoms are influenced by the rate of injection or may tend to disappear after an infusion has continued for some time or upon repeated infusions. Reactions that are similar in character to some of the above mentioned symptoms have been produced by infusing other parenteral fluids (STRUMIA, MCGRAW, and BLAKE, 1943). That some of the reactions may entail liberation of histamine is suggested by the observation that symptoms such as cough and urticaria may be controlled by epinephrine and pyribenzamine or other antihistaminic drugs (JOHNSON, FREEMAN, and MEYER, 1952a; SHAFIROFF and

* Details regarding the preparation and storage of fat emulsion for parenteral use have been published by several authors. Purified pyrogen-free materials are used to whatever extent this may be possible. Three thousand to four thousand pounds pressure is usually desirable when the fat is homogenized by extrusion through a fine orifice. Repeated extrusions are necessary for preparation of a fine uniform emulsion. A stable fat emulsion may be sterilized by autoclaving and stored in gas-tight bottles from which air has been displaced by nitrogen. Storage in the absence of light and at temperatures above freezing have been recommended. The solution is usually made isotonic by the inclusion of dextrose. The average particle size, pyrogenicity and the tendency to cream may be altered by storage and must be checked repeatedly. The technique of preparation of a fat emulsion for parenteral use has been described in detail elsewhere (GEYER, MANN, and STARE, 1948).

MULHOLLAND, 1951). Refinement of the starting materials, great care in preparation and storage of the emulsion, and adjustment of the rate of administration have demonstrated that many reactions can be eliminated when freshly prepared emulsion is administered to human subjects or animals (VAN ITALLIE, WADDELL, GEYER, and STARE, 1952). Data on 1000 infusions to 500 patients recently reported by Stare and associates indicate an incidence of reactions of all types of 12·7 per cent (STARE, WADDELL, and GEYER, 1953).

One of the most perplexing of the reactions to fat infusion is the febrile response that frequently occurs. This response may vary with the same batch of emulsion given to different individuals or with repeated infusions given to the same person. It has been the experience of most investigators that even though fat emulsions are sterile and non-thermogenic when first prepared, yet a thermal response to these same emulsions may occur after a few days or weeks of storage. Explanations for this finding have included bacterial pyrogens, other forms of contamination of the starting materials, decomposition products of the fat or its emulsifying agents, haemolysis produced by the emulsion, and excessive heat production in response to the infusion of a relatively large quantity of a highly calorigenic substance. SHAFIROFF (1951) has emphasized the importance of purification of coconut oil as an important factor in reducing the incidence of febrile reactions. In a series of 750 infusions a temperature response of 1·9° or less was noted in 87 per cent of the patients during the subsequent 24 hr after 1 litre of 10 per cent fat emulsion had been infused; the remaining 13 per cent of the patients had a fever of 2 or more degrees. BECKER and GROSSMAN (1953a) found no correlation between titratable acidity, carbonyl or peroxide content, or particle size and the tendency for a fat emulsion injected intravenously to be thermogenic for rabbits. A recent study of the thermogenic response to fat infusion by LAMBERT, MILLER, and FROST (1951) reported an inability to prepare any fat emulsion dispersed solely by soya phosphatides that was not thermogenic when tested on rabbits, according to the U.S.P. procedure for pyrogen testing. Measures ordinarily effective in eliminating bacterial pyrogens were ineffective. However, an emulsifying agent (Triton A-20) which was shown to reduce the rate of removal of fat from the circulation did prevent the febrile reaction when included in the emulsion. The converse does not seem to be the case since other workers did not find any greater thermogenic response to a fat emulsion following the administration of heparin despite the fact that the rate of removal of fat from the circulation was accelerated (BECKER and GROSSMAN, 1953b).

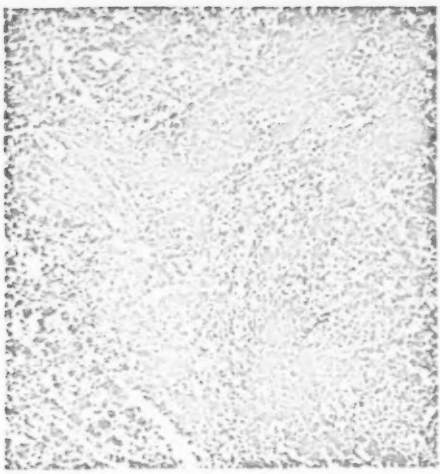
SHAFIROFF, MULHOLLAND, and ROTH (1949) ascribe the fever that may occur with fat infusion to a "sudden plethora of fat." However, as VAN ITALLIE (VAN ITALLIE, WADDELL, GEYER, and STARE, 1952) points out, the thermal response to one batch of fat emulsion may not be elicited in the same patient by another batch. These last-mentioned authors do not believe that the thermal response is due to an excessive production of metabolic heat.

The question as to whether or not the febrile reactions are necessarily due to impurities contained in the fat emulsion or may in some way be related to

the uptake of fat by the tissues remains unanswered. It seems unlikely that bacterial pyrogens are the cause of the thermal response to fat infusion in the majority of instances. Subsequent comments concerning haemolysis and the metabolic effects produced by fat infusion are pertinent to the foregoing considerations.

The effects of daily infusion of relatively large amounts of fat emulsion into animals have been observed for weeks and months. There are also a few reports of patients receiving daily fat injections for several weeks. Puppies receiving approximately 30 per cent of their calories intravenously for 60 to 90 days in the form of emulsified fat continued to grow and have a positive nitrogen balance (MANN, GEYER, WATKIN, SMYTHE, DSAI-CHWEN DJU, ZAMCHECK, and STARE, 1948). A normacytic anaemia did develop and there was a reduction in the plasma cholesterol esters, but essentially no morphological evidence of liver damage. Adult dogs receiving 30 to 40 per cent of their calories as an intravenous fat emulsion likewise remained in good health and nitrogen balance, but became anaemic and had haematuria (MENG and FREEMAN, 1948). There was no functional or histological evidence of liver damage in these animals. Development of anaemia associated with fat infusion has likewise been obtained by others (MCKIBBEN, POPE, THAYER, FERRY, and STARE, 1945; COLLINS, STARE, KRAFT, KINNEY, DAVIDSON, and YOUNG, 1948) who ascribed the anaemia to the emulsifying agents employed. An anaemia in dogs receiving an intravenous fat emulsion was also reported by LERNER, CHAIKOFF, and ENTENMANN (1949). The daily infusion of 2 gm of fat per kgm to rabbits for four weeks was without ill effects as judged by blood pressure, temperature, antigen studies, haematology and histology, according to MENG (1951) who used sodium cholate, soya phosphatide and Span 20 as emulsifying agents. No febrile response was noted to the fat injections unless the emulsion had been prepared for two months or longer; old emulsions were less thermogenic when re-autoclaved. A four-week period of fat infusion did not cause any anaemia in these rabbits.

Relatively few studies on patients have included repeated injections on the same patient to an extent necessary to judge any chronic toxic effects that might develop. HOLT, TIDWELL, and SCOTT (1935) made some observations on children and raised the question as to whether or not the parenteral administration of fat would be contra-indicated in patients with liver disease. A report on one patient who received 1173 calories per day in the form of an intravenous fat emulsion for thirty-six consecutive days, indicated apparent benefit (VAN ITALLIE, WADDELL, GEYER, and STARE, 1952). In another study, patients receiving 1 litre of 15 or 20 per cent fat emulsion daily for four to eleven days yielded no histological evidence of kidney, lung or liver damage in autopsy or biopsy specimens (SHAFIROFF and MULHOLLAND, 1951). Daily injection of 1 litre of a 10 per cent emulsion of olive oil stabilized with phosphatides into senile and debilitated patients for as long as twenty-four consecutive days without obvious anaemia or other ill effects was reported by JOHNSON, FREEMAN, and MEYER (1952a). However, a wide variety of reactions was observed in



(*a*)

(*b*)

Fig. 2. Comparing the distribution of chyle and an artificial fat emulsion in the spleen (MURRAY and FREEMAN, 1951).

(a) Spleen of a rat ten minutes previous to sacrifice received by tail vein 1·0 gm per kg of fat in the form of artificial emulsion. The fat is concentrated in the marginal zone of the red pulp. (Oil red 0 and haematoxylin.. × 120.)

(b) Spleen of a dog immediately after a twenty-minute intravenous infusion of fatty chyle (approximately 0·65 gm per kg total dose). Fat is concentrated in the sheathed arteries and is absent from the margins of the white pulp. (Oil red 0 and haematoxylin. × 110.)

(From *J. Lab. & Clin. Med.* 38 No. 1: MURRAY & FREEMAN, Morphologic Distribution of Fatty Chyle Injections.)

many of the 79 patients receiving single or repeated fat injections (Fig. 1). The tolerance for intravenous fat appeared to differ in various disease states as well as in different individuals or in the same individual given successive injections of fat.

The anaemia so frequently reported to develop in animals receiving daily injections of fat emulsion has been ascribed by CREDITOR (1953) to intravascular haemolysis. This study demonstrated a marked increase in the *in vitro* mechanical fragility of the red blood cells after fat infusion into dogs or human subjects. There was a close correlation between the red cell fragility and the degree of

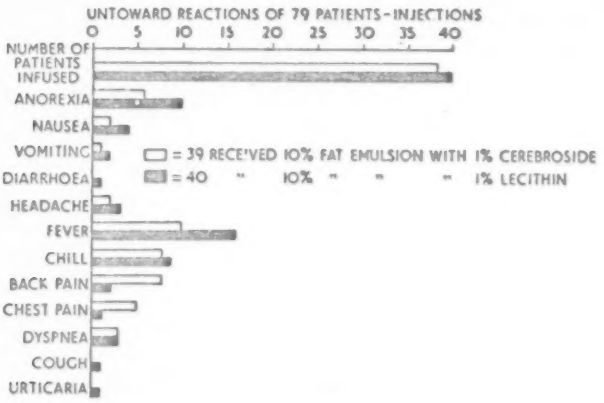


Fig. 1. Illustrating the frequency of symptoms produced by parenteral injections of emulsified olive oil into a group of human subjects (JOHNSON, FREEMAN, and MEYER, 1952a).

lipaemia produced by the fat injection (Table 1). Associated with the injection of fat emulsion as evidence of intravascular haemolysis was an increase in the concentration of plasma haemoglobin and in the faecal excretion of urobilinogen. The increase in mechanical fragility of the red blood cells returned toward normal as the lipaemia subsided. Febrile reactions were most pronounced in subjects that gave evidence of having the most pronounced haemolytic reaction. Other investigators (VAN ITALLIE, WADDELL, GEYER, and STARE, 1952; MENG, 1951) have failed to observe alterations in red blood cell fragility in patients and animals infused with similar emulsions, nor has a different type of emulsion been reported to produce any evidence of haemolytic anaemia (SHAFIROFF, 1951). The extent to which animals or patients may become anaemic in response to repeated fat infusions varies considerably. It is probable that the differences in the observations by various authors concerning the effect of fat infusion on red cell fragility are due to differences in the technique of testing cell fragility or in the rate of fat infusion or the extent to which fat accumulates in the circulation. The increased red cell fragility produced by lipaemic lymph has been ascribed but not proven to be due to its free fatty acid and soap content (JOHNSON and FREEMAN, 1940; JOHNSON, LONGINI, and FREEMAN, 1943).

Table 1.† Studies on human subjects demonstrating the effect of lipaemia produced by fat injection upon plasma haemoglobin and fragility index of the red blood cell (Creditor, 1953)

Exp.	*Min. after infusion*	*Plasma total lipid mg %*	*Plasma Hgb., mg %*	*Fragility index*
A	P*	0	0	31
	0	233	49·5	1,870
	60	96	6·2	2,140
	120	17	5·1	108
	240	0	8·9	87
B	P*	0	0	27
	0	913	23·4	14,500
	60	—	9·6	12,700
	120	484	3·1	10,000
	240	352	6·8	5,500
C	P*	0	0	37
	0	478	1·7	2,625
	60	396	1·7	1,925
	120	230	10·9	268
	180	90	0·5	297

* P = Preinfusion.

† Plasma "total lipid" as used in this table is an expression of the relative turbidity of plasma before and after fat infusion with the preinfusion value as a blank.

Intravascular haemolysis by shortchained glycerides derived from coconut oil and dispersed in gelatin has been reported, while glycerides from the same source containing C_{12} or longer fatty acid chains were not found to be haemolytic under similar conditions (Shafiroff, Mulholland, and Baron, 1952).

At the present time insufficient evidence is available to enable one to judge the extent to which normal or diseased subjects can tolerate repeated daily infusions of 1000 to 3000 calories as emulsified fat for an extended period of time. Post-mortem studies on patients that had been infused with fat have not provided evidence of tissue damage or the accumulation of infused fat in the organs of the body. However, it has been demonstrated experimentally that fat accumulation in the wall of the aorta may be produced by fat infusion in the presence of increased vascular permeability (McAllister and Waters, 1951). The fact that one patient may tolerate an emulsion without any reaction, whereas another person does react adversely to the same preparation, indicates that systemic factors which may be altered in disease states are important considerations that must be better understood before the general applicability of parenteral fat feeding to problems of nutrition can be evaluated. Such an evaluation cannot be made for disease states until an emulsion has been prepared which can be shown to be free of adverse effects on normal individuals receiving the emulsion in similar amounts for a comparable period of time.

IV. Removal of Injected Fat Emulsion from the Circulation

The removal from the circulation and fate of intravenously injected fat emulsions have been studied by many investigators and from a variety of viewpoints. Raper (1913) showed that fat emulsion dispersed with casein and injected intravenously accumulated in the liver. Bloor (1914) found that a fine emulsion of olive or coconut oil was removed from the circulation much more rapidly than injected egg yolk. Removal of injected fat by the reticuloendothelial system was reported by Saxl and Donath (1926). Markowitz and Mann (1930) found a huge increase of fat in the lungs after injection of an artificial emulsion but not after the injection of citrated chyle obtained from a dog fed fat. More of this older literature is mentioned by Rony and Mortimer (1931). More recent studies have largely been concerned with the utilization of fat emulsions by the body. Morphological studies on various tissues indicate that artificially prepared finely emulsified fat is removed from the circulation to a varying degree by the lungs, liver, spleen and possibly by the reticuloendothelial system elsewhere in the body. Fatty deposits in the renal tubes and bile ducts of dogs infused with fat emulsion have also been reported (Dunham and Brunschwig, 1944). The stability of the fat emulsion may influence its distribution in the body (Geyer, Watkin, Matthews, and Stare, 1951). Unstable fat emulsions were found to cause a more marked increase in the fat content of liver, lung and spleen than did stable emulsions, and the extent of extracellular deposition of fat was likewise more marked for the unstable emulsions. The extracellular deposits of fat from unstable emulsions were removed from the tissues more slowly than fat from a stable fat emulsion. Heparin, which increases the *in vivo* dispersion and rate of removal of infused artificial fat emulsion, also reduces the uptake of fat by the spleen (Grossman, Straub, and Decrease, 1952).

Murray and Freeman (1951) compared the immediate fate in rats and dogs of intravenously injected olive oil emulsion with that of injected fatty dog chyle. Ten minutes after injection the artificial fat emulsion was always demonstrable in the capillaries of the lung and in the marginal zone of the splenic red pulp (Fig. 2*a*) while injections of comparable amounts of fat as chyle produced no such changes. Chyle was shown to enter the parenchyma cells of the liver directly, while immediately after injection of the artificial emulsion the fat tended to concentrate in the Küpffer cells. There was a marked accumulation of fat in the sheathed arteries of the dog's spleen after injections of either emulsion (Fig. 2*b*).

Rony and Mortimer (1931) found that the fat contained in chyle derived from dogs fed egg yolk was removed from the circulation of the recipients more rapidly than an injected emulsion of egg yolk. Berry and Ivy (1948) found that injected chyle was cleared from the circulation more rapidly than an emulsion of butter oil stabilized with lecithin. Meng (1952) has reported a more rapid removal of an artificial fat emulsion from the circulation following ligation of the thoracic duct. Some direct transfer of circulating fat across the capillary membrane of intestine and liver is proposed to explain this finding.

A steady rise in the blood fat concentration throughout infusion of a fat emulsion has been reported by several investigators using similar emulsions (NEPTUNE, GEYER, SASLAW, and STARE, 1951; JOHNSON, FREEMAN, and MEYER, 1952a; MENG, 1952), while others using a fat emulsion dispersed in gelatin have reported that the initial rise in blood fat concentration was followed by a decline despite continued administration at an equal or greater rate (SHAFIROFF, MULHOLLAND, and ROTH, 1949). It has been reported that hyaluronidase promotes the absorption of subcutaneously injected fat emulsion (SHAFIROFF, BARON, RECHT, and MULHOLLAND, 1951).

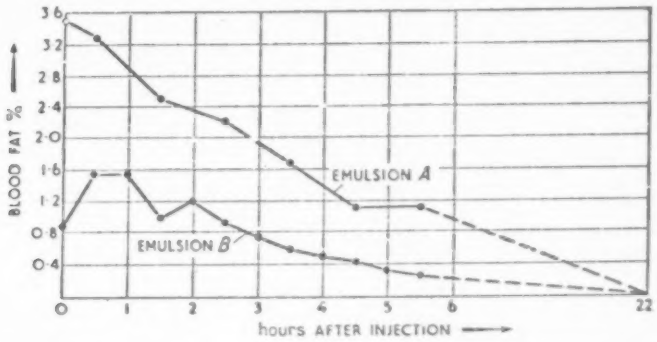


Fig. 3. Average blood plasma curves showing effect of inclusion of Triton A-20 on the rate of disappearance of emulsified fat from the circulation after its injection at a constant rate. Emulsion A contained 15 per cent corn oil, 5 per cent dextrose, 1 per cent soya phosphatide and 2 per cent Triton A-20; emulsion B contained 15 per cent corn oil, 5 per cent dextrose and 1·5 per cent soya phosphatide (LAMBERT, MILLER, and FROST, 1951).

An emulsion of coconut oil stabilized with phosphatide and polyglycerol esters of fatty acids was found to be largely removed from the circulation of dogs, rats and rabbits after 3 to 4 hr, in contrast to mineral oil emulsion of similar dispersion which largely remained in the circulation 6 hr after injection (GEYER, MANN, YOUNG, KINNEY, and STARE, 1948). That the emulsifying agent as well as the material dispersed may influence the rate of removal of infused fat, was clearly demonstrated by the studies of LAMBERT, MILLER, and FROST (1951) who showed that inclusion of a 2 per cent concentration of Triton A-20 in an emulsion of corn oil and phosphatides definitely reduced the rate of removal of injected fat from the circulation (Fig. 3). The effect of the polyglycerol ester was cumulative as the lipaemia was more prolonged with successive injections until it persisted throughout 24 hr.

Metabolic disturbances which alter the fat content of the circulation were found to influence the rate of removal of infused fat both in animals and human subjects. RONY and MORTIMER (1931) reported that factors which effect carbohydrate metabolism influence the rate of removal of injected fat from the circulation. JOHNSON, FREEMAN, and MEYER (1952b) found that patients with glomerulonephritis, and dogs with bilateral ligation of the ureters removed fat

from the circulation more slowly than did their control groups. On the other hand, there was less than the normal elevation of blood fat in cirrhotic patients and dogs with impaired liver function following the infusion of a fat emulsion. Endocrine factors may influence the rate of removal of infused fat since an increased rate of removal of injected fat was demonstrated in patients with thyrotoxicosis, while alloxan diabetic dogs deprived of insulin became markedly lipaemic during fat infusion but showed a remarkable capacity to remove fat from the circulation (Fig. 4).

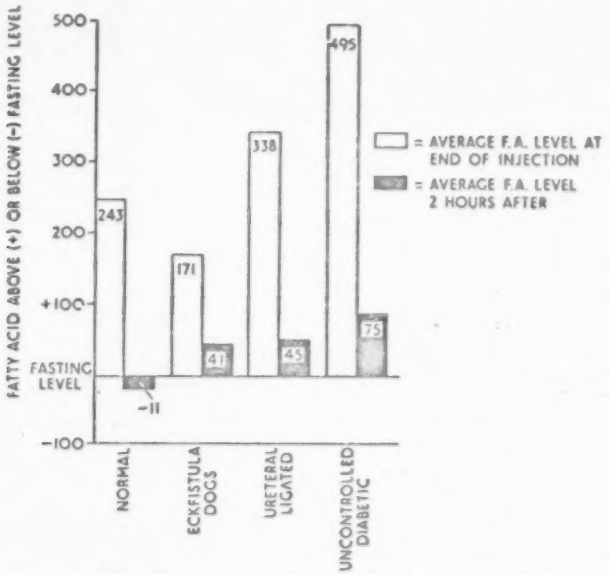


Fig. 4. Illustrating that the removal of fat emulsion from the circulation, following its infusion at a constant rate, may be influenced by experimental procedures that alter the fasting fat content of the circulation (based upon data from JOHNSON, FREEMAN, and MEYER, 1952b).

V. Evidence Concerning the Utilization of Injected Fat Emulsion

One of the earliest studies concerned with the metabolic effect of injected fat emulsion was that of MURLIN and RICHE (1915) who found an increased heat production and a decreased respiratory quotient in dogs for several hours following infusion of a 3 per cent emulsion of lard. BABA (1931) also reported a significant drop in the respiratory quotient following fat infusion. GORDON and LEVINE (1935) demonstrated a reduced respiratory quotient in a healthy infant infused with fat. Prolongation of survival of otherwise starved dogs by the intravenous administration of emulsified olive oil has been reported (NARAT, 1937). Puppies have grown and maintained a positive nitrogen balance for 60 to 90 days while receiving 30 per cent of their calories from injected fat (MANN, GEYER, WATKIN, SMYTHE, DSAI-CHWEN DJU, ZAMCHECK, and STARE, 1948). The prevention of weight loss and the maintenance of nitrogen balance

for several weeks by the parenteral administration of emulsified fat to adult dogs receiving a calorically inadequate diet orally, has been demonstrated repeatedly (McKibben, Ferry, and Stare, 1946; Meng and Freeman, 1948; Meng and Early, 1949). More direct and unequivocal evidence of the oxidation of parenteral fat has been obtained by using tagged compounds. Geyer Chipman, and Stare (1948) injected emulsified trilaurin intravenously in which the carboxyl group was labelled C_{14}. Seventy-one per cent of the C_{14} was recovered in the expired air during $4\frac{1}{2}$ hr following the injection. Lerner,

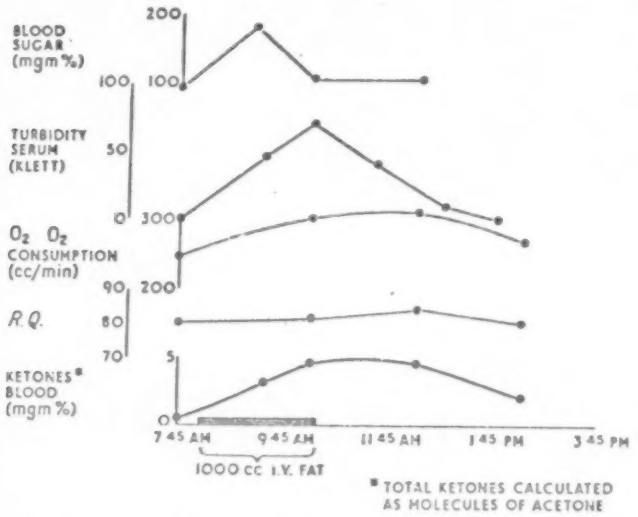


Fig. 5. Showing the blood ketone body increase, fat disappearance rate and other data obtained on a normal male adult subject to whom 1 litre of fat emulsion furnishing 1600 calories was administered intravenously at a constant rate (Neptune, Geyer, Saslaw, and Stare, 1951).

Chaikoff, Entenman, and Dauben (1949) injected emulsified tripalmitin in which the sixth carbon atom of palmitic acid was tagged with C_{14} and recovered in the expired air 50 to 56 per cent of the radioactive carbon within the subsequent 24 hr. Evidence of early oxidation of infused fat by patients has been reported by Shafiroff (1951) who infused patients with an emulsion containing a deuterated fat. The recovery of deuterium in respiratory and urinary water indicated as much as 50 per cent fat oxidation within 24 hr. This author has reported an increased oxygen uptake and heat production associated with a decreased respiratory quotient in fasting subjects following fat infusion. However, only a very slight increase in oxygen consumption and little or no change in respiratory quotient resulted from infusion of 1 litre of a 15 per cent coconut oil emulsion into patients, according to the observations of Neptune, Geyer, Saslaw, and Stare (1951) (Fig. 5). The last-mentioned authors ascribe the increased oxygen consumption that may accompany fat infusion to pyrogenic reactions due to impurities or decomposition products.

The rapid increase in blood ketone bodies 2 to 4 hr after fat infusion must be taken as further evidence of the utilization of infused fat (Fig. 5) (NEPTUNE, GEYER, SASLAW, and STARE, 1951). However, clinically evident ketosis is stated not to occur as the result of injecting emulsified fat intravenously (VAN ITALLIE, WADDELL, GEYER, and STARE, 1952). The urine has been reported as positive for acetone during fat infusion in 20 per cent of the patients that were injected (SHAFIROFF and MULHOLLAND, 1951).

VI. Evidence Regarding the Value of Parenteral Fat as Applied to Man

Reports concerning the use of parenteral fat in human subjects are largely devoted to the extent to which reactions may have occurred and to laboratory data regarding the effect of the fat emulsion on the blood, urine or function of organs, particularly the liver. However, there is abundant proof that the calories contained in parenteral fat can be utilized as a source of energy. It can scarcely be questioned but that a concentrated source of available calories in an isotonic fluid may be of great value to some patients whose calorie intake is inadequate over extended periods of time. The usefulness of such an emulsion cannot be assessed until all toxic effects not inherently characteristic of emulsified fat have been eliminated from preparations used on normal subjects and patients. Complete absence in some individuals of any adverse reactions associated with fat infusion make it apparent that reactions are not a necessary accompaniment of the injection of emulsified fat into the circulation.

There is meagre clinical and laboratory evidence of beneficial effects of parenteral fat administered to malnourished patients. One such patient, given daily fat infusions for 36 consecutive days, was referred to earlier in this account (VAN ITALLIE, WADDELL, GEYER, and STARE, 1952). Elsewhere it was reported that four seriously ill patients receiving daily infusions of 1 litre of 15 per cent fat emulsion for 10 to 21 days were able to maintain their body weight during these infusions (NEPTUNE, GEYER, SASLAW, and STARE, 1951). There was a reduced nitrogen and potassium excretion by a patient on a calorically inadequate diet when coconut oil emulsion was administered intravenously (GORENS, GEYER, MATTHEWS, and STARE, 1949). Some weight gain and other evidences of improved nutrition have likewise been reported in debilitated patients receiving 500 to 1000 cc of 10 per cent fat emulsion for 24 days (JOHNSON, FREEMAN, and MEYER, 1952a). Elsewhere it is stated that a positive nitrogen balance can readily be restored in patients with severe inanition using a fat emulsion combined with other forms of parenteral nourishment (SHAFIROFF, 1951).

VII. Discussion

The extent to which disease may influence the response of the organism to injected fat cannot be satisfactorily evaluated until the normal response has been established. A sparing action of oral fat on body nitrogen under conditions of simple caloric deficiency has long been recognized (LUSK, 1931). The extent

to which protein is catabolized during starvation is influenced by the fat content of the body, according to the experiments of VOIT (LUSK, 1931). It was also shown by VOIT that the ingestion of varying quantities of fat by well-nourished, fasting animals had very little influence on protein metabolism. A starving dog burned 96 gm of body fat, and after receiving 100 gm of fat orally, the animal burned 97 gm. As LUSK points out, the conditions of metabolism were identical in the two instances. The ingestion of fat did not alter the extent to which protein was broken down in the body. Similar observations were made by BARTMANN (LUSK, 1931), who found that fat given up to 150 per cent of the energy requirement was readily absorbed but only spared protein to a maximum of 7 per cent. Much fat administration was sometimes associated with increased elimination of nitrogen in the urine and stools. This last mentioned effect calls to mind the increased bile pigment excretion reported to occur in association with high fat diets (LOEWY, FREEMAN, MARCHELLO, and JOHNSON, 1943).

The increased protein catabolism associated with certain acute processes such as burns or fractures is generally regarded as endocrine in origin and not readily modified by a high caloric intake. The extent to which malnutrition and debility, as they exist in many chronic diseases, may be modified by extra calories remains to be determined. There is no satisfactory basis for evaluating the extent to which infused fat may spare body fat. However, since the infused fat is utilized, and if no catabolism is stimulated by its infusion, one may assume that it spares body fat to the same extent that oral fat does. The extent to which injected fat will spare nitrogen is bound to vary in different conditions. Measuring the effect of fat infusion on nitrogen or potassium excretion is undoubtedly an inadequate basis for judging the nutritional value of infused fat. It should also be mentioned that fat emulsions may have a nutritional value quite apart from their caloric content, as a vehicle for nutritionally essential fat soluble constituents (STARE, WADDELL, and GEYER, 1953).

The digestion and absorption of fat normally requires many hours (MUNK and ROSENSTEIN, 1891; BLOOR, 1914). Although the peak of lipaemic curve usually occurs 4 to 6 hr after fat ingestion, it is not uncommon for the serum to be lipaemic 12 hr or longer after a fat-rich meal. A lag of 1 to 3 hr is usually observed following fat ingestion before the lymph in the thoracic duct shows an increased fat content. The fat present in lymph during fat absorption is largely neutral fat. Neutral fat is normally removed rapidly from the circulation as evidenced by the relatively low neutral fat content of fasting blood.

The rapid infusion of emulsified fat, even though it be tolerated so far as one may discern, is hardly to be regarded as a procedure that simulates normal body processes. In this regard, fat, by its very nature, represents a special problem beyond that presented by the digestion products of either carbohydrates or proteins. Water solubility is not a factor in the transport or diffusion of the amino acid or simple sugar, but special mechanisms must be involved if dispersion and solution of fat in aqueous media is to take place. While the emulsification of fat that occurs prior to infusion provides for some of the body's requirements for handling fat, yet other factors probably none

the less essential, though less apparent or well understood, may not be called into play in normal sequence with the abnormal egress of fat into the circulation.

It is generally acknowledged that hyperglycaemia normally stimulates insulin secretion by the pancreas and thereby accelerates the mechanism of sugar removal from the circulation. Imbalance between sugar ingress into the circulation and the hormonal response to its presence in excessive amounts leads to disturbances in the homeostatic mechanism that may be manifested by hyper- or hypoglycaemia or both. Endocrine mechanisms that may be stimulated by amino acid absorption are not clearly defined and many tissues of the body appear to possess an inherent capacity to remove amino acids from the circulation. An excess of either sugar or amino acids may be excreted in the urine. There is no immediate effective excretory mechanism for the disposal of excessive amounts of fat that may be present in the circulation. Removal of aggregates of colloidal substances from the circulation by the reticulo-endothelial system is a general mechanism for coping with substances that fall in this category. The extent to which this mechanism is invoked by fat infusion appears to depend upon a variety of factors. Colloidal stability, particle size and composition are factors that have been demonstrated to influence the removal of fat from the circulation. Studies concerning the effect of heparin and protamine injection on alimentary lipaemia (BROWN, 1952) suggest that a dispersing factor may be elaborated normally as part of the response to fat absorption. Factors regulating this mechanism may be of consequence in determining the body's response to injected fat. Further study of the extent to which digestion and absorption normally influence fat transport will help in arriving at some appreciation of the extent to which parenteral fat must initially be treated as a foreign substance and whether or not lipaemia in itself will stimulate the normal mechanisms that provide for the dispersion and uptake of fat by the storage cells of the body. In short, a more complete appreciation of the factors concerned in normal fat transport, storage and metabolism will enable a more intelligent approach to the problems surrounding the use of parenteral fat.

VIII. SUMMARY

The general subject of parenteral fat administration to animals and man has been reviewed. From consideration of the various aspects of the problem it appears that the current status of this subject is reflected by the following statements:

(1) Fine, stable emulsions of fat have been prepared and injected repeatedly intravenously into animals and human subjects.

(2) The fat contained in these emulsions is largely removed from the circulation within 3 or 4 hr following injection. The rate of removal and distribution of the fat are influenced by systemic factors as well as by the composition and characteristics of the emulsion itself.

(3) These emulsions may provoke a variety of abnormal reactions, or no adverse reactions whatsoever, in the recipients.

(4) The thermogenic response that frequently accompanies the infusion of emulsified fat does not appear to be due to bacterial pyrogens in the more refined preparations.

(5) There is ample evidence that emulsified neutral fat injected intravenously can be oxidized by the body. However, the extent to which fat infusion may alter the metabolic mixture undergoing oxidation by the body requires further clarification.

(6) Limitations in the present understanding of bodily mechanisms concerned in fat transport impair the progress of attempts to arrive at a suitable fat emulsion for parenteral use.

(7) The parenteral administration of fat is not yet perfected to the extent of being generally applicable to undernourished individuals.

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2

SOLUTIONS OF SOAP-LIKE SUBSTANCES

G. S. Hartley

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2

SOLUTIONS OF SOAP-LIKE SUBSTANCES

G. S. Hartley

I. Introduction

Over the last fifty years the study of solutions of soap-like substances has received an apparently disproportionate amount of the attention of physico-chemical research workers. Two causes have contributed to this, but historically three phases of activity can be distinguished.

The first cause is essentially intellectual. Paradox and conflict have always been a challenge to the imagination. Ionic substances show one extreme of behaviour in regard to solubility, having affinity only for the most highly polar and associated solvents. Paraffins, as their name (from *parvum affinum*) conveys, are extremely solvent-indifferent and are consequently rejected by solvents whose molecules have a high degree of mutual attraction. A long paraffin chain with an ionic group covalently attached to its end constitutes a molecule in which these extremes of solution behaviour must coexist. Solutions of such substances are obviously peculiar. The mechanism by which the conflict is resolved in these peculiar solutions has naturally aroused the curiosity of many research workers.

The second cause is essentially practical. The liquid state of matter is the one most obviously and conveniently adapted to chemical processing, because it is easily and compactly containable, stirrable, and transmissible. Water is, by many orders of magnitude, the most abundant of liquids, but its solvent powers are very specialized. A wide range of substances requiring processing in the living plant or animal and in the home or factory, are not dissolved, and perhaps not even wetted, by water. The soap-like molecules, having a foot in either camp, are the liaison officers. as it were, which resolve this difficulty. Soapy water will emulsify oils which pure water will not dissolve, will wet materials on which pure water would remain in discrete drops, and will suspend finely divided solids which would float in air cushions on pure water.

The second cause operated first. Practical interest has nearly always preceded academic interest, although most of our academicians since the early days of the Royal Society have tried to hide themselves from this fact. The ancient art of soap-boiling—i.e. the hydrolysis of fatty glycerides in strong hot soda followed by the salting out of molten soap—posed a number of peculiar problems in the relationships of two or more liquid phases, and gave the first stimulus to the development of present knowledge of these solutions.

The early investigations of the modern period concerned themselves therefore rather naturally with concentrated aqueous solutions. Even at concentrations

insufficient to cause separation of a second, anisotropic, phase, these aqueous solutions showed many abnormalities of behaviour.[1] The study of these abnormalities made progress more quickly when yet more dilute solutions were examined, but, in very dilute solution, the "natural" soaps (i.e. salts of fatty acids of some 10 to 18 C atom content) are so extensively hydrolysed as to obscure the more typical abnormalities.

Academic tendencies dictated therefore a transfer of attention to similar salts in which the end group is the anion of a strong acid. REYCHLER's investigations of fatty sulphonates[2] appeared to be independent of the modest technical applications of the related TWITCHELL reagent.[3] There followed a period where academic investigations of newer compounds at least kept pace with industrial development. Extension of work to compounds differing in the location of the ionic group was again approximately contemporary and independent in the academic and technical fields.

Then, however, we entered an era of very rapid industrial expansion. Seeing the potentialities of synthetic compounds in variety limited only by available manpower and expenditure, rival industrial concerns poured on to the market a host of new emulsifiers, wetting agents and detergents, giving rise, amongst other problems, to an almost insuperable one for analytical control. Not only has the "surfactant" industry, as it is called in the U.S.A., achieved a state of chaos in fifteen years unequalled by that achieved by dyestuffs over a period four times this long, but it produces products in many cases of low purity, all of which are very effective in interfering with the separation of other compounds.

The common feature of all surface active agents is the existence in a single molecule of regions of contrasting solution-tendencies which are sufficiently separated to act, as far as their mutual attachment permits, independently. The semi-natural soaps were for a long time the only members of this class, and the first synthetic compounds followed a closely similar pattern, only the nature of the small ionic head group being changed, from $—CO_2^-$ to $—SO_3^-$ and $—O—SO_3^-$ and $—\overset{+}{N}(Me)_3$, etc. The general name "paraffin chain salts" was a convenient one. Now, however, it is not general enough, and it is better to restrict its use to the simpler types than to misuse it. "Surface active agents" is widely used, but might be held to include many finely divided homogeneous solids which we do not wish to consider. It includes also the horrible tautology "active agent," although this could easily be avoided by replacing "agent" by "substance." Some years ago the author[4] proposed the convenient word "amphipathic" to describe the essential property of conflicting sympathy and antipathy, and this word has been fairly widely accepted in the scientific literature. We shall use "amphipathic substances" for the general class, and "paraffin chain salts" for the simplest and most characteristic members.

We are concerned here primarily with the nature of solutions of amphipathic substances. The important technical aspects of surface activity as such will be considered only as they are affected by the nature of the solutions.

II. Solutions in General

To understand the nature of these peculiar liquid solutions, we must understand first the nature of more typical ones.

A true solution is spontaneously formed when two previously separated phases of different composition are brought together. In the gaseous state of matter solutions are universal and complete. Gases consist of widely separated molecules moving at great speed, occasionally colliding, and contained only by the boundaries of the space allowed to them. Nothing can stop gases mixing, because nothing can stop a moving molecule from moving into the wide spaces between other molecules.

In liquids, each molecule is all the time within the powerful force fields of its close neighbours and is therefore self-containing. Thermal movement shows itself, by producing frequent interchange of positions, in the property of fluidity. A molecule in a liquid, in other words, must always have close neighbours, but they will not always be the same neighbours. Since change of neighbours occurs, change of kinds of neighbour can also occur when different substances in the liquid state are brought together, i.e. the liquids mutually dissolve, but since the forces of attraction between the possible pairs of molecules may be widely different, not all liquids are completely miscible.

Liquids are mutually soluble to an extent depending on the difference between the force of attraction between unlike molecules and the mean of those between the like pairs. In general the attraction between unlike molecules is less than the mean of the attractions of like molecules, and so the attractive forces are best satisfied by association of the molecules, like with like, in separate phases. This tendency is opposed by the mixing effect of thermal agitation, increasing with temperature. Consequently only rather extremely dissimilar liquids are not completely miscible; those which are not completely miscible usually become more soluble in one another with increase of temperature, and at least one of the liquids of an immiscible pair has molecules of very high polarity or transient bonding tendency.

One can recognize different degrees of mutual solubility of liquids even when they mix in all proportions. Thus, water is completely miscible with both methyl and propyl alcohols, but it is easy to see that it is "only just" miscible with the latter—a very small addition of benzene or salt, for example, will cause a large degree of separation of water and propyl alcohol. It is convenient to give quantitative expression to this fact, and this is most conveniently done by means of the partial vapour pressure. For liquids ideally soluble, the vapour pressure of each, plotted against mol fraction, is a straight line. The less mutually soluble are the liquids, the more do these lines become convex upwards. Only for occasional pairs, such as chloroform and ether, do we find convexity downwards, indicative of mutual attraction being greater than the mean of self-attractions.

In the crystalline state the movement of molecules is much further restricted. Not only does a molecule always have close neighbours, but also neighbours in fixed relative positions and orientations, and this means that, in the vast

majority of cases, the neighbours remain individually the same. Usually, therefore, no solution occurs in a crystalline solid. The highly efficient mutual attraction necessary to overcome the disrupting effect of thermal agitation and fix the molecules in a crystal lattice can only be attained by bringing the most strongly attracting parts of molecules into the best relative positions. Consequently, the geometry of molecules becomes of great importance in the crystalline state.

The solubility of a crystalline solid in liquids is determined by the same factors which govern the mutual solubility of liquids, with other factors in addition. These determine its tendency to dissolve in anything at all, even in a liquid of very similar composition. (The liquid of most similar composition is of course the molten substance itself, but in this the solid is either completely miscible or entirely insoluble, according to whether we are above or below the melting point.) The further we are below the melting point and the higher the latent heat of fusion, the less is the tendency to dissolve at all, and this factor is superimposed, when we come to be concerned with the solubility in a dissimilar liquid, on the factors affecting the solubility in this liquid of one chemically similar to our chosen solid. The melting point particularly, and, to a less extent, the latent head of fusion, are dependent on much more specific properties of substances, including molecular geometry, than are the mutual dissolving tendencies of liquids.

Failure to distinguish the crystalline and liquid factors has been responsible for many misconceptions and mistakes. Let us consider a few illustrative examples.

Benzene, although of low solubility in water, is much more soluble than diphenylmethane. Solubility of a slightly soluble liquid is always decreased by increasing the size of the molecule, while keeping the same chemical type. Diphenyl, smaller by the central methylene group, is, however, even less soluble than diphenylmethane. If we were to conclude from this fact that the methylene group has a marked water attraction, we should be making a quite wrong interpretation. The dominant factor in this case is that diphenyl (m.p. 70°C) is a crystalline solid having much less tendency to dissolve in anything than diphenylmethane (m.p. 26°C).

Phenol is much more soluble in water than benzene despite its higher melting point, and this is of course attributable to the very hydrophilic —OH group. The difference is so great that we might expect two —OH groups on the benzene ring to confer mutual miscibility with water. Water is miscible with fused resorcinol under pressure, and at room temperature resorcinol is much more soluble than phenol. Hydroquinone, however (ca. 5 per cent), is less soluble in water than phenol (7 per cent). This is not due to some cancellation of the attractive effects of the para groups, but to the melting point having soared to 170°C (resorcinol, 110°C; phenol, 41°C). As a crystalline solid of high melting point, hydroquinone is not very soluble in any liquid. It is, as expected, more soluble in water than in most other liquids.

The solubility of salts is influenced by much more specific factors than

operate in the case of unionized compounds. In the first place, the force field of ionic groups is of a very extreme type. As a very powerful electrostatic field, it is capable of orienting polar molecules, and, as a highly divergent electrostatic field, is capable of attracting the oriented dipoles. The mutual attraction of oppositely charged ions, however, is also very high, and we find that generally electrolytes are only soluble in highly polar compounds, and water is of course an outstanding solvent for them.

The majority of common salts are also crystalline, and specific geometrically operating factors are again of great importance. Consequently we find that salts have a very low solubility except in certain special solvents, including water, and that in these special solvents the solubility is very specifically dependent on the particular combination of ions. As in the example of phenol and hydroquinone mentioned above, we must not draw erroneous conclusions from the insolubility of some particular salt; for example, the insolubility of barium sulphate is not to be attributed to any unusually poor satisfaction of intra-molecular forces between a barium ion or a sulphate ion and water molecules, but it is to be attributed to an exceptionally stable crystalline arrangement, which makes barium sulphate of course a quite generally insoluble substance.

So specific and powerful are the intra-molecular forces involved in the solution of an electrolyte, that they exert a dominating influence where they arise, with the result that very frequently the influence of other factors tends to be ignored. It is not generally realized that modification of the behaviour of salts when one ion is also a large organic molecule is apparent in solutions of many organic electrolytes, although in not so extreme a form as in the special substances which we are concerned with in this article. For example, we are so accustomed to thinking that introduction of salts into water depresses the solubility therein of unionized organic substances, and we are so accustomed to thinking of simple organic solutes such as sodium benzoate and trimethylamine hydrochloride as salts, that we are inclined to forget that concentrated solutions of them in water already show some modification of salt effects by organic solvent effects. In low concentrations, the salting out is dominant, but in high concentrations the organic influence, so to speak, may prevail over the electrolyte influence, and in fact concentrated solutions of these relatively simple salts are much better solvents than water for a number of organic substances.

Solutions of two liquids which are nearly ideal in the sense above defined—such as benzene, cyclohexane; ethylene dichloride, ethylene dibromide—are very simple in internal structure. The molecules are freely interchangeable and distributed at random. The properties of such solutions are simple means of the properties of the pure liquids, provided that the right sort of mean is considered.

This is true of the solvent properties of the mixture for a third substance, provided it is not too soluble, as for example a crystalline substance of high melting point, or a vapour at less than its saturation pressure, or a permanent

gas. The logarithm of this (low) solubility is a linear function of the mol fraction composition of the mixed solvent. If the third substance is very soluble in one only of the constituents of the solvent mixture, attempts to saturate the solvent with it result in the complicating circumstances of separation into two liquid phases, and the behaviour is in consequence considerably more complicated.

We are most usually concerned with the solvent properties of a mixture of a very good solvent and a very bad solvent. Very frequently, for example, a bad solvent is added to a solution in a good solvent in order to precipitate some wanted product in synthetic chemistry. The precipitation is quite consistent with the logarithmic linear relationship above mentioned. If, for instance, a crystalline organic substance is 10,000 times as soluble in alcohol as it is in water, we should expect it to have only 100 times the water solubility in an equimolar solution of alcohol in water, which is approximately 75 per cent alcohol. Consequently the addition of only 30 per cent of water to the saturated solution in alcohol will result in almost complete precipitation.

Very few solutions are ideal, and in the vast majority of non-ideal solutions the molecular forces between a pair of unlike molecules is less than the mean of those between pairs of like molecules. In such cases the application of simple theory to prediction of the solvent properties of the mixture will lead us to expect that the mixture will always be a rather better solvent than it would have been if the solvents themselves had formed an ideal solution. This prediction can be made even on the assumption that the molecules in the non-ideal solvent mixture are still distributed at random. Actually this will not be the case, because whenever molecules are, as it were, really reluctant to mix (but not sufficiently reluctant to outweigh the mixing effect of thermal agitation) they will not be distributed quite at random, but will tend to come together in small, transient clusters, like to like.

In the consideration of any non-ideal behaviour of solutions, it is not easy to distinguish the effects directly due to unequal distribution of molecular forces, and the effects directly due to the clustering tendency, which is itself one of the effects of the primary cause. In certain particular cases there may be fairly clear distinction. For example, benzoic acid dissolved in benzene exists to a very large extent as definite molecular pairs, formed by hydrogen bond association through the carboxyl groups. The thermodynamic behaviour of benzoic acid in benzene fits in quantitatively with the view that the benzoic acid forms an equilibrium state between single and double molecules. If we take, however, alcohol in water, there is very good evidence that the alcohol molecules are, to some extent, grouped together, but the association is very much looser than in the example just quoted, and has no clearly defined limit. Alcohol molecules are doubtless found adjacent to one another more frequently than would be the case in a purely random distribution, but the association will be in the form of pairs, triplets, etc., the clusters increasing in average size as the alcohol concentration is increased.

The effect of this non-random distribution on the solvent properties of the

mixed solvent is to give the mixture yet higher solvent power than would otherwise be expected. The solvent power, however, is still much closer to a logarithmic mean than to an arithmetic mean. Fifty per cent alcohol is still a very poor solvent compared with alcohol for organic substances which are almost insoluble in water, but the effect of the non-ideal solvent mixture is clearly illustrated by a comparison of 50 per cent methanol, ethanol and propanol. The solvent power of these mixtures relative to that of the pure alcohols increases markedly in that order,[5] with increasing tendency to cluster.

With simple solvents we find that the solvent power of the mixture is only an arithmetic mean of the solvent powers of the two separate solvents when the solvents remain separate. That is to say, only when they are immiscible.

III. Solutions of Amphipathic Substances

The saturated paraffins are about the least soluble of all liquids in water. Nearly all simple organic substances are more soluble in other liquids than they are in a vacuum, that is to say that the volume concentration at equilibrium in solution, even in a poor solvent, is greater than the volume concentration in the saturated vapour. Paraffins are so extremely insoluble in water that they provide the only known exceptions to this generalization. The mutual attraction of water molecules is so great, and so completely incapable of being satisfied by association with paraffin instead, that the paraffin molecule is, so to speak, squeezed out of solution to a very extreme degree.

What happens therefore when we introduce into water a substance in which an extremely hydrophilic ionic group is covalently attached to a fairly large paraffinic group is a matter of some theoretical interest, as well as of course of immense practical importance. We have brought together inescapably in the same molecule the two widest possible extremes of solvent reaction. In the behaviour of the salts of the lower fatty acids there is nothing very remarkable. The intra-molecular forces between the ionic group and the water molecules is so great that the intrusion of a few carbon atoms saturated with hydrogen is of little account, but when we come into the molecular weight range usually classed as soaps, namely from about 10 carbon atoms upwards, the characteristic and peculiar behaviour of amphipathic substances becomes evident. We have now introduced so much paraffinic matter into each ion that the water molecules can, so to speak, hardly fail to notice the intrusion. On the other hand, their attraction for the ionic group is so strong that it cannot be so lightly relinquished. If another liquid, immiscible with water, and miscible with paraffin, is present, the obvious answer to the problem is the absorption of the soap ions into the interface, the paraffinic tails lying within the oil phase and the ionic heads in the aqueous phase. This results in the increased facility of gross mixing of the two phases, so as to provide sufficient area to accommodate all the absorbable ions. Otherwise expressed, the interfacial tension is reduced to a very low value, and extension of the interface by disruption of one of the phases is facilitated.

Where, however, no such surface offers itself for satisfaction of the amphipathic property by absorption, there is a tendency for spontaneous formation of separate paraffinic phases in an anisotropic state of division. In other words, the amphipathic ions tend to distribute themselves not randomly throughout the solution, but in clusters, of which the paraffinic tails form the interior and the water-attracting ionic groups the surface.

It is important to appreciate that it is the enormous mutual attraction of water molecules and their attraction for ionic groups which are the important factors in this process. The mutual attraction between the paraffinic groups in isolation is not very great, and is in fact less than with practically all other groups known to organic chemistry. We have to attain molecules of fairly considerable size and very regular shape in the paraffinic series before we find substances which are crystalline at room temperature, and the substances are all more volatile than other carbon compounds of similar molecular weight. The association of amphipathic ions in aqueous solution is therefore to be considered, from the molecular force point of view, as an association of water molecules leaving clusters of non-water attracting groups of the largest possible dimensions in order that there should be least intrusion amongst the water molecules. Consistent with this view, the partial volume of water in the solutions decreases when aggregation occurs while that of the paraffinic moiety increases.[6] We do not expect, therefore, nor do we find, any elaborate organization of these aggregates, or micelles, as they are generally called.

IV. Normal Paraffin Chain Salts

We will first of all consider the simplest members of our peculiar class. These include the true soaps, and are the compounds in which we have a simple unbranched saturated paraffin chain with a relatively small ionic group at one end. Many of the newer amphipathic substances which are of greatest use in industry have a less simple geometrical form, and their behaviour in solution is rather more complicated. It is convenient, therefore, to consider the extreme and simple type of substances first.

Where such substances are soluble in water, they are usually very soluble. If the solubility exceeds something of the order of 1 part in 1000, it will probably attain a value of some 50 or more per cent. Generally speaking, these salts have a positive temperature coefficient of solubility in water, and as a consequence of these two properties, the curve of solubility against temperature has a rather extreme form, showing a normal degree of temperature dependence in the low concentration region, changing over fairly abruptly into a very steep ascent in the high concentration region.[7] One may in fact almost speak of a critical solution temperature, irrespective of concentration. This circumstance is itself evidence of aggregation in these solutions.

Other evidence for aggregation is provided by the facts:

1. Depression of water vapour pressure in paraffin chain salt solutions at high concentration is considerably less than in the case of a simple electrolyte of corresponding concentration.

2. The overall mobility of the paraffin chain ions in the electric field is abnormally high. While the total equivalent conductivity is less than that of a simple electrolyte at corresponding concentration, when multiplied by the transport number to give the paraffin chain mobility, this is found to exceed very considerably the value obtaining at infinite dilution. The only conceivable mechanism of this is that the paraffin chain ions are grouped together, so that the same electrical force is operative against a diminished viscous resistance.

3. The solvent power of an aqueous paraffin chain salt solution for organic substances of low water solubility is much greater than that of an ordinary water-miscible solvent at similar concentration, and in fact the amount dissolved in excess of that dissolved by water only is almost proportional to the paraffin chain salt concentration over a very considerable range.

This last point is one of the most important lines of evidence, and one which is of considerable technical interest; it has also aroused a very considerable interest in academic circles of recent years. Its significance is clearly envisaged in the closing paragraphs of our introductory discussion. As far as solvent power is concerned, the solution of the paraffin chain salt in water behaves almost as though it were not a solution, but a gross mixture of two immiscible liquids. Nevertheless, the solutions are usually optically clear. One can only conclude that the paraffinic phase is extremely finely divided; that is to say that the paraffin chain ions are aggregated together into clusters very much larger than the solute molecules, but still smaller than the wavelength of light.

I have above deliberately avoided the use of the word "solubilization," which I consider, besides being a very ugly word, to be a misleading and unnecessary one. It implies that the solute has suffered some active chemical change, which of course is not the case. There is nothing peculiar about the solute, but only about the solvent, and the peculiarity in the solvent power of the mixed solvent is a peculiarity in degree only, and not in kind. We shall have more to say about this subject later.

Further evidence of aggregation is provided by the existence of a rather sudden transition in all accurately measurable physical properties of the solution in the neighbourhood of a fairly well defined concentration characteristic of the salt concerned, and particularly dependent on the length of the paraffin chain. The form of this change in physical property depends, of course, on the nature of the property examined. If, for example, we measure the total volume of a solution containing a constant amount of water as a function of the amount of paraffin chain salt dissolved in it, we have of course a continuously increasing volume, but the rate of increase suffers a change at this critical concentration. Consequently, if we plot not the total volume, but the differential of the total volume with respect to salt amount, the resulting function, the partial specific volume, suffers a fairly abrupt stepwise transition, from one more or less constant value to another. The total volume divided by the total amount of salt, i.e. the apparent specific volume, shows an increase commencing suddenly, but approaching its new maximum more slowly.

The types of curve are shown in Fig. 1. It is important, when comparing evidence from quite different kinds of measurement, to ensure that functions of the same mathematical type are compared. Thus, the equivalent conductivity curve should be compared, not with dV/dC, but with V/C.

The significance of this rather abrupt change in properties was first appreciated by BURY in explaining the rather less marked, but related changes, occurring in aqueous solutions of butyric acid.[8] He pointed out that it was

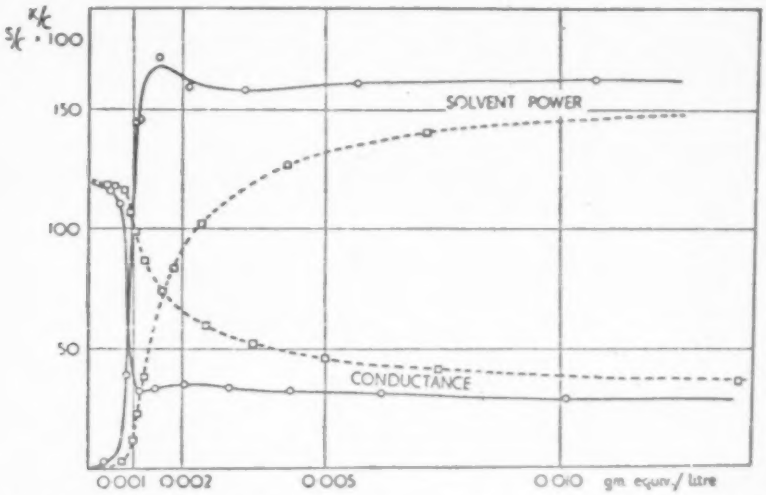

Fig. 1. Comparison of variation of conductivity and solvent power with concentration to show necessity of comparing functions of the same type.

S = solubility of trans-azobenzene at 25°C in aqueous cetyl pyridinium chloride of concentration C. Data from HARTLEY.

κ = specific conductivity of cetyl pyridinium bromide at 35°C. Data from HARTLEY, COLLIE, and SAMIS.

Full curves are for $\frac{dS}{dC}$ and $\frac{d\kappa}{dC}$

Broken curves are for $\frac{S - S_0}{C}$ and $\lambda = \frac{\kappa}{C}$

to be expected from the application of simple mass action considerations to aggregation if the assumption was made that the aggregates consisted of fairly large clusters of molecules, with no appreciable concentration of clusters of intermediate magnitude. In its extreme form we might imagine the size of the clusters to be infinite, in which case of course we should be simply dealing with a phase change. For example, we should get even more sharply marked changes, of the same type as those observed in the clear aqueous solutions of paraffin chain salts, if we plotted the properties of a mixture over a range of additions of solute which went beyond the saturation point. The abrupt change would commence, of course, as soon as saturation was reached. These considerations were further elaborated by later workers, but it is not possible to make, from the degree of abruptness of the changes, any quantitative estimate of the

degree of aggregation, except that the aggregates must be much larger than any occurring in simple solutions. The important point is that the sudden change would be very much blurred by the presence, in appreciable concentration, of intermediate states of aggregation.

We are dealing with a mechanism of aggregation, which can operate in the case of a large aggregate, but which does not, at any concentration, favour a smaller one. We must also bear in mind that we are dealing with an equilibrium system, and that not only are these large aggregates preferred to smaller ones, but they are also subject to some limiting factor. This is a consideration which must not be overlooked if we are to arrive at a satisfactory picture, although one of the most commonly postulated types of aggregate, namely the lamellar aggregate, with the paraffin chains arranged parallel to one another, provides no satisfactory mechanism for this limitation.[9] As the writer has elsewhere expressed it,[10] this picture is very easily drawn, but the difficulty is to stop drawing it; one may fairly ask whether Nature herself does not find a corresponding difficulty. The basic mechanism of aggregation arises from the very strong tendency of the self-attracting water molecules to reject the paraffin chains, while retaining the ionic groups. If we postulate an aggregate, with the paraffin chains forming an essentially liquid or chaotic interior, and the ionic groups forming the outer surface of the resulting spherical particle, we do obtain a picture which gives a satisfactory explanation, both of the absence of small aggregates and of the limitation in size of the large ones.

Two chains, forming a pair, will cause comparatively little reduction, per chain, in the extent to which water molecules are undesirably separated. A third chain, lining up with the first two, will cause a rather bigger reduction in the number of water molecules separated. Comparatively little further energy advantage is obtained beyond this point, and is only realized for a small number of chains when they are lying parallel to one another. When parallel, they still expose a large surface of contact between paraffin and water and moreover require to be in a particular disposition relative to one another in order to secure what energy advantage is obtained. In the large spherical aggregate, on the other hand, the whole exterior is dominated by the presence of the extremely hydrophilic ions, so that the maximum energy advantage is obtained, and the orientation of the chains in the interior is subject to very much less restriction. The large spherical liquid aggregate is therefore favoured. If, however, this is to increase in size, then either some ionic groups must be submerged in the paraffin interior, against which operate the very strong forces between the ions and water, or the spherical aggregate must assume some anisodimensional form in which a greater restriction on the orientation of the chains is imposed.[11]

Direct experimental evidence in favour of this liquid arrangement is provided by the very high solvent power of aqueous solutions of paraffin chain salts above referred to. Only liquids, in which the arrangement of molecules is chaotic are generally good solvents. Solutions in the solid state, as has already been pointed out, are very unusual. The remarkable features of the solvent

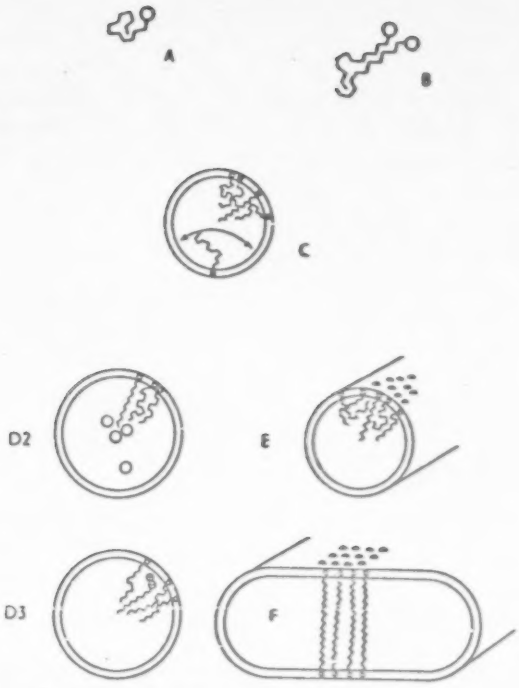

Fig. 2. At *A* is depicted the generally accepted disposition of a single paraffin-chain ion in water, the chain being crumpled on itself so as to separate the fewest possible water molecules.

At *B* is a hypothetical pair of ions. Not much more configurational latitude can be allowed to each chain and the fact that they must assume a close relationship of configuration more than compensates for this. The energy advantage is very small as the arrangement at best reduces only slightly the number of water molecules separated.

At *C* we have a schematic section of the spherical micelle shown incompletely filled by paraffin-chain ions. The arrows show the configurational latitude allowable which is greatly increased compared with *A* and *B*. The "arrowed" chain cannot of course assume any configuration in the space indicated irrespective of the behaviour of neighbouring chains, but neither could it in paraffin liquid. It has, in the micelle, about one-third of the space available in paraffin liquid under the same conditions of nearest neighbour restraint. The energy advantage is nearly maximal, as the ionic groups in the outer shell constitute an almost completely hydrophilic skin.

To gain greater energy advantage by further increase in size of aggregate there are three apparent possibilities:

D, increase of size while remaining spherical. This requires either

(1) a "hole" in the middle which cannot be more than a few C atoms in size;

(2) a paraffin-soluble additional solvent which can populate a small central region exclusively. This explains why dissolved substances increase the micelle size;

(3) a submergence of ionic groups in the paraffinic portion of the micelle which is perhaps permissible with the more organic-soluble ion combinations—e.g. more probable with cetyl pyridinium iodide than with chloride.

E, increase in one dimension only, i.e. through a prolate spheroid towards the extreme of a cylindrical rod, shown in section including the two minor axes, in which the only difference from *C* is that the shell is more densely populated with ions so that a smaller proportion of chains can, at any one time, reach the centre. This forces a "less than random" parallelism between chains. Much the most probable deviation from *C*.

F, increase in two dimensions, i.e. through an oblate spheroid towards the extreme of a lamella. Since the chief outer surfaces are parallel and as far apart as possible, the chains are forced into a more than random parallelism and therefore the arrangement requires much greater restriction which will not normally occur unless the local energy advantage of crystalline packing becomes dominant. The arrangement is in fact that of an undissolved crystal.

power of paraffin chain salt solutions are (1) that this solvent power is very great, far greater than in a corresponding concentration of a molecularly diluted solvent, such as acetone or alcohol, and (2) that it is very general. A large liquid micelle provides an explanation of both these peculiarities. The solvent power is great because the additional solvent is not really diluted in terms of molecular magnitudes. It is very general because the additional solvent is still a liquid. The solubility of a non-polar substance which is a crystalline solid of sufficiently high melting point to have a rather low solubility in any solvent is determined mainly by its solubility in paraffin, i.e. it dissolves in the paraffinic interior of the micelle without sufficiently changing the properties of the latter to have any serious effect. This has been established for the case of azobenzene in solutions of cetyl pyridinium chloride[12] and for azobenzene and naphthalene in potassium laurate and dodecyl ammonium bromide.[13] Generally the crystalline substance is rather less soluble in paraffin chain salt than this theory would predict, and the soap is a noticeably less good solvent than the cationic paraffin chain salt.

Where the crystalline organic substance has a partly polar character and is in fact slightly amphipathic itself, as with dimethylaminoazobenzene,[14] the solubility may be rather greater than predicted, due doubtless to partial incorporation in the micelle in the same orientation as the paraffin chain ions themselves.

Where we are dealing with a liquid hydrocarbon, the solubility is quite definite and a clear distinction can be drawn between the emulsion and the optically clear solution. No abnormal viscosity or anisotropy of the solution results. Presumably the swollen micelle still remains spherical and the limitation of solubility is now imposed by the fact that the micelle cannot be very extensively swollen before the innermost portion of it ceases to be penetrated by paraffin chains. As soon as an inner region containing several molecules of added hydrocarbon only develops, this region is no longer molecularly diluted, and has the full vapour pressure of the liquid in the pure state.[15] The solubility limit is therefore reached. The solubilities of several hydrocarbons have been investigated[16] and also the vapour pressures over solutions which are less than saturated in the liquid hydrocarbon.[17]

Further evidence in favour of the liquid arrangement of paraffin chains is provided by the nature of the density changes during aggregation. In all cases that have been investigated, the partial volume of the paraffin chain salt increases during the aggregation, while that of the water decreases.[6] Not only is the change in this direction, but, in the case of the paraffin chain salts of fairly long chain length, the partial volumes in solution are actually greater than the partial volumes in the solid state. A striking example is provided by cetyl pyridinium chloride, which is denser than water in the crystalline solid form, but solutions of which are lighter than water.[18] This is a very abnormal state of affairs for an electrolyte solution, because the hydration of ions results in a compression of the water molecules in the hydration layer, and a consequent increase of density. The solution of all simple electrolytes in water is

accompanied by a reduction of total volume. The explanation of the reverse phenomenon in the case of the paraffin chain salts must lie in the behaviour of the paraffin chains themselves, and is clearly due to a change from the parallel arrangement in the crystalline solid to the chaotic liquid arrangement in the solution aggregate. So important is this line of evidence that one may perhaps be excused for stressing once more the significance of a property so easily measured as density, but which is so often not measured before a lot of more complicated and less easily interpreted experiments have been undertaken.

It does not, of course, follow directly from this evidence of the liquid nature of the aggregate that the aggregate is spherical. There are, however, two more direct lines of evidence in this connection. Firstly, we have the direct determination of size of the aggregates. Measurements of osmotic pressure have not been fruitful because the equilibrium between aggregates and single ions is so facile, and the penetrating powers of the single, almost linear ion, so great that it is practically impossible to find a satisfactory membrane. The most direct experimental evidence is therefore that obtained by diffusion measurements,[19] which had to be made in a swamping excess of simple electrolyte in order to overcome diffusion potential effects, which would otherwise make the results almost impossible to interpret. The results of these measurements agreed very satisfactorily with the spherical micelle theory, the radius being slightly greater than the length of a fully extended chain.

The other line of experimental evidence was first used in an entirely opposite direction, and is that provided by the x-ray diffraction camera. When strong solutions of paraffin chain salts are examined with this instrument, a moderately clear diffraction ring is obtained, indicative of a long spacing, which decreases with increasing concentration of solution.[20] There is also present a less well-defined outer ring, indicative of a short spacing approximating to that to be expected between parallel paraffin chains. These diffraction rings were first interpreted in terms of orthodox crystalline structure, and it was assumed that the long spacing must represent that between corresponding *planes* in oriented lamellar micelles. The explanation, however, did not go far enough back into the fundamentals of the subject. All that we can really conclude, in what must necessarily be the equivalent of a powder photograph, is that there is a repeating distance in the solution between corresponding arrays of diffracting atoms, but these need not necessarily be constant distances between *plane* surfaces. The alternative explanation in terms of the spherical micelle theory was made by HARTLEY,[21] who pointed out that in concentrated solution the very high electric charges of the spherical aggregates would cause them to be disposed in approximately a three-dimensional honeycomb pattern. The nearest neighbour distances between corresponding parts of these spheres can be calculated (see below) in terms of the total volume concentration and the radius of the spheres, and it was found that the variation of long spacing with concentration could be quite simply and satisfactorily accounted for, assuming spheres of constant radius rather greater than that of a fully extended chain.

This view has now been accepted by experts in the analysis of x-ray diffraction pictures. Not only is the long spacing satisfactorily accounted for, but also a fainter diffraction line indicative of an intermediate spacing. The short spacing has received less attention. It would be desirable to know in the first place whether it is any stronger than would be expected in an amicroscopic emulsion of the corresponding straight-chain hydrocarbon alone. In these, local parallelism of chains is to be expected on purely geometrical grounds. When we say that the interior of the micelle is liquid we do not mean that it is more chaotic than the bulk liquid hydrocarbon itself.

A further line of evidence on the structure of the micelles is provided by light-scattering phenomena.[23] There is no appreciable scattering of light by particles which are considerably smaller than the wavelength of light, especially when they are composed of material differing very little in refracting power from the continuous medium around them. When at least one dimension of the particles exceeds considerably the wavelength of light, light is scattered and the intensity of the scattered light, as a function of the angle between scattered and incident light, depends on the number of dimensions in which the wavelength of light is exceeded. Aqueous solutions of the majority of soluble paraffin chain salts alone at concentrations of only a few per cent, which is still well above the critical concentration for the majority of the salts we are concerned with (and where therefore it is clear that most of the paraffin chain ions exist in the aggregated state) are optically empty. Therefore the only evidence to be derived from light-scattering is that the particles have no dimension greater than about 500 Å. In very high concentration, however, or frequently in the presence of a high concentration of simple electrolytes, the solutions become appreciably turbid and the measurement of scattered light intensity now gives us not only information about size, but also some information about shape. Light is scattered in all directions from the transmitting beam, but the variation of intensity with angle can be shown to be different for different shapes of particle. DEBYE concludes that we are dealing in these solutions with very elongated particles which are, however, in the nature of thin rods rather than thin plates, that is to say, that only one of the three cartesian dimensions exceeds the wavelength of light, and all that can be stated about the others is that they are considerably less than the wavelength. It is of interest in this connection that HARTLEY, in considering the factors involved in micelle formation, concluded that the liquid sphere was the most probable, but that if any departure from the sphere form occurred, it was more likely to be in the direction of a prolate than of an oblate spheroid,[24] since the former allows considerably more latitude in the geometrical configuration of the paraffin chains. He considered in fact that the balance between the sphere and the prolate spheroid was a rather delicate one. The prolate spheroid enables a more closely packed ionic exterior to be presented to the water. Maximum packing density is attained around the minimum circumference when the eccentricity is still fairly small. If the conditions disturb the balance in favour of the prolate spheroid, the mechanism of intrinsic limitation

of aggregation is lost and the extension to long rod-like particles is almost necessary. Their mean length will be statistically determined by concentration.[25]

The diffusion measurements cited above as being the most direct evidence in favour of the spherical particle are obviously very difficult to reconcile with any other picture, but they were made only on optically clear solutions in which light-scattering measurements would not indicate anything else. It is therefore most probable that the particles are essentially spherical in the simpler pure solutions where these are optically clear and of low viscosity and that in some cases, particularly on the addition of simple electrolytes, the balance is disturbed in favour of the rod-like particles which produced the form of light scattering observed.

The presence of very long rod-like particles is likely to produce another observable effect, namely an abnormally high viscosity. Not only is an abnormally high viscosity apparent in the case of certain specific solutes, even at fairly low concentrations, but it is a viscosity of a qualitatively anomalous type. In particular, elastic recoil is very evident in such solutions, and has been sometimes noted in extremely low concentrations.[26] It seems quite impossible to account for this elastic behaviour without postulating the presence of very elongated particles. Not only must they be elongated, but they must be capable of being stretched elastically. In the case of similar elasticity produced in solution by certain proteins, particularly mucin in, for example, the excretion of the parotid gland which is responsible for the familiar "ropiness" of saliva, it is generally considered that the elasticity of the particles responsible is essentially a reversible folding of the protein chain, which of course has complex potentialities of internal association. Such a mechanism in the case of elastic solutions of paraffin chain salts seems to be outside the possibilities of their chemical structure. If, however, we are dealing with very long rod-like particles, the interior of which is essentially liquid, we can see at once an entirely different mechanism of elastic recoil. Such a particle can clearly be stretched. As it is stretched, the hydrophilic groups in its surface are necessarily separated farther apart, the interfacial tension is therefore necessarily increased, and the rod tends to contract to its original shape, after the manner of the crawling worm rather than a spring. Elastic contraction is not inconsistent with indefiniteness of equilibrium length because in it we are concerned with contraction to a resting length per unit amount of paraffin chain content—or lateral expansion to a fixed maximum.

This structural elastic effect in paraffin chain salt solutions is notably sensitive to very small changes. The earliest example is provided by ammonium oleate solutions, which are elastic, in contrast to solutions of the sodium or potassium salts, which have normal and not very viscous behaviour. Ammonium oleate is, however, a very feeble example in comparison with others that have later been reported, and where the differences are even smaller. For example, the strong and saturated cetyl sulphonic acid, which forms perfectly normal solutions with the majority of bases when the salts are soluble, forms

very highly elastic solutions between about 60°C and 80°C when the base is aniline or *para*toluidine, but solutions without any trace of elasticity when the aniline carries a methyl group on the nitrogen atom. The elastic solutions retain the general solvent properties which are a clear indication of an essentially fluid structure of the micelle, but the introduction of too much micelle-soluble material destroys the elasticity. This is not surprising, in view of the fact that it must clearly increase the size of the micelle and therefore provide a means of reducing the separation between the hydrophilic groups alternative to the formation of an elongated particle.

Optical anisotropy should of course be detectable in the solution of liquid micelles when these are in the filamentous condition, since, although the interior of such micelles contains fluid paraffin, it cannot of course be entirely anisotropic. Since all the chains in a cylinder terminate in the surface, they will lie more nearly radially than if they did not do so. The deviation from random distribution will be small, and the anisotropy therefore feeble. It has, however, been demonstrated under sheer by SCHERAGA and BACKUS[27] working with cetyltrimethyl ammonium bromide in the presence of potassium bromide. No birefringence is detectable in solutions without the added simple salt. It is estimated that the mean length of the rod-micelles with 0·15N KBr is 1200 Å and, with 0·4N KBr, 4000 Å.

The lamellar micelle is still considered by many writers to be the most probable structure despite its inherent improbability, and a good deal of experimental evidence against it. It is well, therefore, to summarize this evidence.

It was first held to be the only explanation of the x-ray diffraction patterns. The long spacing was considered to be that between corresponding planes and its increase with decrease of concentration to be due to the inclusion of more water between the parallel ionic faces. Whatever the lateral dimensions of the lamellae, much more water must be situated between the exposed paraffinic cylindrical faces than between the ionic planes. The water between these planes, although many molecules thick, is nevertheless characteristically determined by total concentration. This distribution of water is very peculiar and no exponent of the lamellar micelle theory has suggested any explanation. A further difficulty arises if we consider the increase of spacing due to the introduction of a paraffinic soluble solute—this is disproportionately great and the exponents of the theory have now to assume that the water accommodated between the ionic layers is increased by the incorporation of solute between the paraffinic layers. "Curiouser and curiouser!"

The spherical micelle provides a satisfactory explanation of these phenomena. We have only to postulate that the micelles are of constant size in the pure solution, the radius being a little more than the length of an extended chain, and arrange themselves as far away from their nearest neighbours (due to coulomb forces) as is geometrically possible. Introduction of a solute within the micelle increases the size disproportionately because it is mainly in the interior and permits a greater radius to be attained.

If the chain length is r and its effective cross section in solution is A, the pure micelle has paraffinic volume $\frac{4}{3}\pi r^3$ and a content of $\frac{4\pi r^3}{3A}$ chains. Suppose we now introduce a paraffin-soluble solvent in volume fraction ρ of the paraffin volume. If uniformly distributed, the radius of the micelle will not change and its content of chains must be decreased. This would result in an increase of number of micelles and therefore a reduced long spacing. Since, however, the micelle strives to have as hydrophilic an exterior as possible, it takes advantage of the presence of the solute to grow, by leaving solute alone to occupy the innermost region. This type of growth cannot occur indefinitely, as this would lead to a phase separation, but it is clearly possible for the size to increase by a fraction of a chain length. Even a single molecule at the centre can produce a profound effect. Take for example a 15 C atom chain, for which r is effectively 20 Å, giving a paraffinic interior for the micelle of volume 33,500 Å^3 and containing therefore about 70 chains. A molecule of liquid benzene occupies a sphere of approximately 150 Å^3 or of radius 3·3 Å. A *single* benzene molecule at the centre therefore increases the micelle radius to 23·3 Å and its volume to approximately 52,900 Å^3. The paraffinic volume is thus increased by rather more than 50 per cent and the number of micelles reduced accordingly. In this case the addition of as little as 0·5 per cent of benzene (by volume on the paraffin volume) would produce a 15 per cent increase of long spacing. In fact, of course, the effect is not so extreme as this, because the paraffin-soluble solute will not be confined to the centre. We introduce this calculation only to show that there is nothing surprising, on the spherical micelle theory, in a disproportionate effect of addition of paraffin-soluble substance.

The lamellar micelle theory requires an unexplained and improbable behaviour of the water. It also requires an unsatisfactory and improbable arrangement of the paraffin chains. Unsatisfactory because a cylindrical surface, of indefinite extent, of paraffinic matter is exposed to the water, improbable because a strict parallel alignment of the chains is usually postulated. This is a much more restricted arrangement than the liquid one and will not be favoured therefore unless there is an energy advantage to be gained. This does not normally arise unless the closer packed pattern of the crystal is attained. Coupled with there being no evident mechanism for the limitation of size of the lamella aggregates, we conclude that they do not exist, except in indefinite size as crystals.

Not only have we this strong theoretical argument against the lamellar micelle, which is essentially a crystalline micelle, but we have the experimental evidence, as previously quoted, both of solvent power and density, that the micelle is not crystalline, but essentially liquid. Furthermore, there is direct evidence from diffusion measurements, as well as indirect evidence from x-ray diffraction, that the size of the micelle in clear solutions is about that predicted by the micelle theory, and that in the turbid solutions there is the evidence of light scattering measurements that the micelle is elongated in one dimension only.

V. Electrochemistry

We have so far approached the subject of the state of solution of paraffin chain salts from the viewpoint of the general theory of solubility. Measurement of solvent power and density could have established the present theories almost unaided but most of the earlier evidence was based on electrical measurements. This was due to the ease and accuracy of these measurements and to the primary interest at the time in electrical charge on colloidal particles as the main factor in determining their properties.

Further emphasis was placed on the electrical conductivity of paraffin chain salts by the classic discovery of McBain[28] that, over a limited range of concentration, the equivalent conductivity of the salt *increases* with increasing concentration. This was explained by the decreased resistance to movement through a viscous liquid of a close packed group of ions as compared with that of the separate ions. Since the total electrical charge, and therefore the total force acting in a given potential gradient, does not, to a first approximation, change on aggregation, the combined ions move faster than the separate ones.

This was, as later discoveries showed, the right explanation of the wrong fact. When studies were extended to salts of strong acids, in which no complications from hydrolysis alkalinity obscure the behaviour at great dilution, it was found that the equivalent conductivity first deviates from normal behaviour at a very much lower concentration than that in which the rising conductivity is observed.[29] The first deviation is sudden and the sudden change is usually a decrease. If we accept that the aggregation of ions of like charge is accompanied by an attraction of a considerable fraction of unlike also to the highly charged aggregate, a fall of total conductivity is not surprising. Clearer significance can be obtained if measurements of transport number are made as well as conductivity, so that the equivalent conductivities of the ionic constituents can be plotted separately.

When this is done it is found that, at the critical concentration, the equivalent conductivity of the paraffin chain ions does indeed rise,[30] and above its infinite dilution value, a fact which can only be explained by the decreased resistance consequent on aggregation. The fall of total conductivity is caused by a very rapid fall of the equivalent conductivity of the counter ions, this quantity frequently assuming a negative value, because more counter ions are moved backward by their attachment to the paraffin chain aggregate than move forwards separately. This phenomenon is naturally more extreme when divalent counter ions are concerned.

In the later rise of equivalent conductivity in much higher concentrations, a phenomenon of much smaller magnitude and not invariably present, it is found that the paraffin chain conductivity, which rose at the critical concentration, is now falling, whereas the counter ion conductivity is rising again and frequently more steeply. The earlier explanation cannot therefore be correct.

The fall of paraffin chain conductivity, once aggregation is effectively complete, is presumably the normal effect of increasing concentration, particularly marked in the case of a very multivalent electrolyte. If aggregation had not

occurred, the paraffin chain conductivity would have continued to fall slowly as in typical uni-univalent electrolytes. If aggregation could have been present and complete at infinite dilution, we should have found evidence of a very high conductivity in this limiting condition, accompanied by a very rapid decrease with increasing concentration. In the transition region we pass over from the first curve to the second fairly abruptly. (The transition nature of the process would be better evident if the differential quotient $d\kappa/dc$ were plotted instead of the conventional $\lambda = \kappa/C$.)

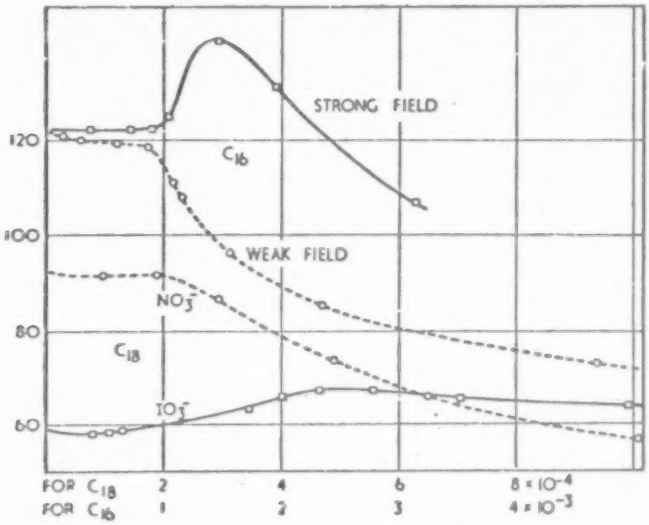

Fig. 3. Comparison of equivalent conductivities versus concentration for octadecyl pyridinium nitrate (broken curve) and octadecyl pyridinium iodate (full curve), from data of GRIEGER, KRAUS *et al.*; and for cetyl pyridinium chloride at low field strength (broken curve) and at 250 kV per cm for 8×10^{-7} sec. (full curve), from data of MALSCH and HARTLEY. Twenty-five units added to figures for latter salt.

If, for a given paraffin chain salt, we could initiate aggregation at successively lower concentrations we would expect to obtain the first evidence of aggregation by a rise of equivalent conductivity, followed by a later fall. Obtaining our reduction of the critical concentration, however, by increase of chain length we might expect this prediction to be defeated by the effect of necessarily increasing initial charge on the rate of decrease of conductivity of the aggregate with increasing concentration. It has, however, recently been shown experimentally that the total equivalent conductivity can indeed rise at the critical concentration if this is low enough and if the counter ion is of low mobility in the separate condition, so that it makes less contribution throughout. The effect is just evident with hexadecyl pyridinium iodate but is very pronounced with the octadecyl compound.[31] The corresponding chlorides do not show this transient increase. The λ–C curve for octadecyl pyridinium iodate is reminiscent (although the concentration is of course lower) to the extrapolated curve for

cetylpyridinium chloride in a field of very great strength (250 kV/cm)[32] in which the depressant effect of the associated counter ions is partially removed by the high velocity ion being removed from its associated cloud of counter ions (Wien effect) and consequently being able to shed a portion of those actually attached.

It is the general depressant effect of salt concentration on mobility, very great in the case of these multivalent systems and almost unpredictable along DEBYE-HÜCKEL lines, that makes the accurate tools of conductivity and transport number measurements of disappointingly little value. It is almost impossible to distinguish the effects of actual, if transient, attachment of counter ions to the micelle from "ionic atmosphere" effects. The problem has been discussed theoretically in a detailed manner by the writer elsewhere.[18] An experimental attack was also initiated in which it was suggested that the effect of "all other" ions in the aggregate on the tendency for association of one of them could be estimated in terms of the ζ-potential, which is calculable from the measurable conductivity of the aggregated paraffin chain ions. This potential determines the ratio $\left(\frac{\varepsilon\zeta}{e^{\bar{\kappa}T}}\right)$ of the concentration of (univalent) counter ions at the micelle surface to the mean concentration in the bulk solution. The study showed a reasonable agreement with measurements of the apparent pH displacement when an indicator ion is absorbed in the micelle.[33] (Apparent pH displacements of nearly 2 pH units are frequent, when a paraffin chain salt is added to a buffered indicator.) This line of approach might be worthy of further development, but workers in this field have recently tended to use electro-chemical measurements as a qualitative tool only.

It has been mentioned that, after the transition to micelle solution is complete, the fall of paraffin chain conductivity is accompanied by a rise of that of the counter ions. If the fraction of counter ions incorporated in the micelle remained constant, the second observation might simply follow from the first, because the retrograde movement of this constant fraction decreases and might do so more rapidly than the forward movement of the free ions. This effect alone, however, could not account for an increase of total equivalent conductivity. This phenomenon seems to mean that the free counter ions become more mobile with increase of concentration or that more of them become free. The writer is inclined to the view that the explanation is to be sought in the structure of the solution combined with the very great ionic atmosphere effects. A dilute micelle solution consists of localized highly conducting regions separated by regions almost devoid of ions. Increasing concentration may then enable the conducting regions to make easier contact with one another. The conductivity of a suspension of metal spheres in non-conducting oil would clearly increase much more rapidly than the concentration of metal. If this view were correct, the conductivity of a colloidal electrolyte and a simple one might be more than additive, a prediction recently put to experimental test with only partial success.[34]

Whatever the detailed behaviour of counter ions around and on the micelle

surface, it is clear that the charge on the paraffin chain ions themselves is, *per se*, a factor tending to oppose aggregation. The dispersive effect of this charge is mitigated, directly and indirectly, by the counter ions. If these are increased in number by adding a simple salt, aggregation of the paraffin chain ions is facilitated and therefore occurs at a lower concentration. Several experimental studies have confirmed this.[35]

VI. Salts with Partly Aromatic Chains

The long normal paraffin chains are still derived almost entirely from vegetable and animal sources. Stimulated mainly by greater ease of manufacture from more readily available raw materials, industry has produced many technical wetting agents in which the straight paraffin chain has been replaced by alkyl substituted aromatic nuclei. The alkyl substituents are themselves frequently branched, and are derived by condensation of unsaturated hydrocarbons from the petroleum industry. Typical examples of this class are di-isobutyl naphthalene sulphonate and dimethylhexyl benzene sulphonate.

Few of these compounds have emulsifying properties comparable with those of normal paraffin chain salts having an equal number of carbon atoms. They find their main and very effective application as wetting agents. The nature of the solutions of these compounds has been comparatively little studied, partly because the abnormalities in solution typical of the paraffin chain salts proper do not arise until concentrations higher than those of ordinary technical interest are attained. One must expect a reduced tendency to form aggregates because, generally speaking, the length of the hydrophobic parts of these molecules is considerably less than that of normal paraffin chain salts having the same number of carbon atoms. Moreover, the aromatic nucleus is less extremely hydrophobic than saturated paraffin. It has been concluded from conductivity measurements that the benzene nucleus is equivalent to 1·5 methylene groups.[36]

Some examples of this class have been included in solution studies, particularly of the solvent power for water-insoluble substances, and it is evident from these that a similar type of aggregation does occur, but to considerably less extent.

VII. Salts with Two Chains or Non-terminal Ionic Groups

Examples of this class are the well-knowr Aerosol series for example, Aerosol OT, which is the sodium sulphonate of dioctyl succinate, the sulphonic acid group being attached to one of the two central carbon atoms of the succinic acid group. The octyl residues are dimethyl hexyls. These compounds are readily made by condensation of sodium sulphite with the corresponding esters of maleic acid.

The Aerosols are the most widely used compounds of this class, but they suffer, in common with all carboxylic esters, from fairly rapid hydrolysis in alkaline solution. This is not shared by the Tergitol compounds, which are formed by aldol condensations of petroleum products, followed by formation of

the sulphate esters of the alcohol finally produced. Tergitol 7 has a saturated branched chain of in all 17 carbon atoms, 9 carbon atoms being found on one side of that carrying the sulphate ester group, and 7 on the other.[37]

Other compounds of the same general type have been subject to academic investigation. A series convenient for study, but not technically available, is composed of the sulphonates of dialkyl ethers of dihydric phenols.[38] HARTLEY investigated the interfacial tension between cyclohexane and carbon tetrachloride and aqueous solutions of these compounds, and found that, while in very low concentration they were less active than similar compounds with all the aliphatic carbon atoms in the same chain, the interfacial tension continued to decrease beyond the concentration at which in the latter case it had become almost constant.[39] Whereas with simple paraffin chain salts interfacial tension does not fall below about 2 dynes per cm, in the case of the sulphonates of 1,2 or 1,3 dioctyloxybenzene, interfacial tensions as low as 0·04 dynes per cm were measured; doubtless even lower interfacial tensions are attainable, but self-emulsification in the stalagmometer capillary made their measurement impossible. The reason for this behaviour is as follows. Two chains attached to an ionic group are almost as easily rejected by water into a pre-existing oil phase as is a single chain of the same total length, but owing to the fully stretched length of the hydrophobic part of the ion being much shorter, the formation by chains of a separate paraffinic phase is rendered considerably more difficult. Consequently a much higher concentration of unaggregated ions can be attained.

WINSOR has studied the behaviour of a series of N-tetradecyl sulphates,[40] including all positions of the ionic group from carbon atoms 1 to 7. He found that the critical concentration in water rose from $1{\cdot}7 \times 10^{-3}$ N with a terminal ionic group to 16×10^{-3} N with a central ionic group. RALSTON and collaborators studied, by conductivity, a series of dimethyl dialkylammonium salts.[41] The abrupt fall at a critical concentration was present, but the concentration was much higher than in single chain salts of the same number of C atoms. For the dioctyldimethyl salt it was 0·03 N, whereas extrapolation of data for trimethylammonium salts would put the figure for the corresponding pentadecyl trimethyl at 0·002 N. The didecyldimethyl salt had a critical concentration of 0·0025 N as compared with 0·0004 N for the nonadecyltrimethyl.

It is of interest that, in this series, the didodecyldimethyl ammonium chloride was found to be sufficiently soluble for conductimetric investigation of the critical concentration, found to be *ca.* 0·0001 N. This must be the largest number (26) of aliphatic carbon atoms carried into solution by one ionic group! The critical concentration was here heralded by an initial rise of equivalent conductivity, an occasional phenomenon commented on in the electrochemical section. The total ionic charge of the paraffin chain ions in a "double C_{12}" micelle must of course be about half that in a "single C_{12}" micelle, and much less of course than in a "single C_{16}" micelle which would form at the same critical concentration. The retarding effect of counter ions is therefore much less.

Compounds of the double chain type have been examined for organic solvent power in aqueous solution, and show this typical property of the paraffin chain salts, although considerably less strongly than the corresponding simple salts.[42]

In regard to solubility, the branched, or double, chain salts differ considerably from the single chain salts. Firstly, they have a rather limited solubility as optically clear solutions, but when present in larger concentrations in water, they appear to form spontaneous emulsions of a more concentrated phase in a more dilute one. Precise light scattering measurements do not appear to have been carried out on these solutions, but the absence both of streaming double refraction and abnormal viscosity indicate that the suspended phase is neither strongly anisodimensional nor anisotropic. HARTLEY has reported an interesting example of this phenomenon which demonstrates its complete spontaneity.[33] He found that the potassium salt of the monosulphonate of the di-n-octyl ether of resorcinol can be obtained, by evaporation of water from a water-glycerine solution, in massive pyramidal crystals. When transferred to pure water these redissolve with a turbid "halo" and, if the total concentration exceeds about 1 per cent, the solution remains turbid.

The second difference is that they are much more readily soluble in organic solvents than corresponding simple paraffin chain salts. This organic solubility is so pronounced that it operates against the effectiveness of the compounds as emulsifying agents for aromatic or polar liquids. They are, however, extremely active emulsifiers for saturated aliphatic hydrocarbon, in which liquids alone they are less soluble than in water.

The most probable explanation of these solubility peculiarities is to be found in the crystal structure of these double chain salts being a much less stable arrangement than that of the corresponding simple salts. Consequently, they have a greater tendency to dissolve in all solvents, but this tendency is not equally manifest in water because of the greater difficulty of forming the normal type of aggregate which contributes to the very high solubility in water of the simple paraffin chain salts.

VIII. UNIONIZED AMPHIPATHIC SUBSTANCES

The essential characteristic of the amphipathic salts is the presence of two strongly opposed solution tendencies covalently united in the same molecule. The most extreme behaviour is naturally found with a combination of the most extremely opposed types of solvent reaction, namely the saturated paraffin on the one hand and the strongly ionized group on the other. There are, however, many strongly hydrophilic groups which do not carry an overall electric charge, and if sufficient of these are present attached to one end of a paraffin chain, a solution in water results, having many of the peculiarities of a solution of a paraffin chain salt. Such substances are good wetting agents and fairly good emulsifying agents, and in high concentration they show considerable solubility for organic substances. A considerable technical development of this type of substance has been undertaken because amphipathic substances of this type

have one very great advantage over the ionic compounds in that their behaviour is almost independent of ionic impurities in water. The deficiencies of soap in hard water are of course well known. Many of the synthetic compounds have avoided the worst of these disadvantages because salts have been obtained whose calcium salts are not insoluble. Nevertheless, although introduction of divalent cations to a solution containing them may not result in precipitation, it still has a profound influence on the interfacial properties. The unionized compounds, on the other hand, are almost completely indifferent to low concentrations of simple electrolytes in water.

The most obvious choice for a non-ionized substitute for the ionic group would be a head group containing a number of hydroxyl groups, as for example the glucoside of a fatty alcohol. Compounds of this type have been made but, owing to ease of manufacture, the choice has usually fallen on a polythylene oxide chain, obtained by the condensation of ethylene oxide with a fatty acid or alcohol or an alkyl substituted phenol, etc. Condensed glycerol has also been used in place of ethylene oxide.

There seems no doubt, mainly from solubility evidence again, that aggregation is present in solutions of the non-ionic compounds.[43], [44] Exact studies of aqueous solutions of these compounds are rendered difficult by two features. Firstly the difficulty of obtaining pure compounds, since extensive and indefinite condensations are involved in synthesis and the compounds are not crystalline. Secondly the non-availability of electrical methods of investigation.

Solutions of these compounds are of particular interest, from the solution structure point of view, at high concentrations, and will be referred to later in the section dealing with multi-phase systems.

IX. More Complex Amphipathic Substances

We have dealt briefly with a number of different types of amphipathic substances which can be considered developments of the original paraffin chain salts. It will be realized that none of these variations is exclusive. Where the hydrophobic portion is partly aliphatic and partly aromatic, it can also be branched, and two short chains can be attached to the same ionic group. At the same time, it is possible to combine either or both of these deviations from the simple chain substance with a head group which does not rely for its water attraction on the presence of a free ion or ions. Finally, all these variations can be brought together in the same molecule, and we may have for instance two fairly short paraffin chains attached to a polyhydroxylic head group which also contains an ion. In the pursuit of surface active agents specially adapted to particular purposes, compounds with this very complex structure have been made.[45]

Recently there has been an approach to the surface active substances from workers in the field of polymer chemistry. "Polysoaps" can be obtained, for example, by the partial quaternarization of polyvinylpyridine. The main skeleton of the polymer is hydrophobic. Water attracting groups are the basic tertiary nitrogens and the less frequent ionized quaternary nitrogens. Although

the degree of amphipathy of the repeating unit, considered alone, is not great, the usual exaggeration of properties in polymers comes into play. If a polymer unit is more soluble in benzene than in water, the polymer itself will be benzene-soluble and water-insoluble, because the energy of transfer is multiplied on polymerization, while the thermal energy remains the same. Similarly a unit rather feebly surface active will become, on polymerization, very strongly so. Absorption is naturally slow, but correspondingly tenacious. The polysoaps are not such good emulsifying agents as the monosoaps but are much better emulsion stabilizers. Aqueous solutions of polysoaps are solvents for water-insoluble organic substances which dissolve in the hydrophobic interior of the coiled single-molecule micelle.[46] In the case of benzene, but not of other C_6 hydrocarbons, the viscosity of the aqueous solution is raised by the solution of a small amount of hydrocarbon. A larger amount of benzene and any amount of other hydrocarbons lowers the viscosity. It seems probable therefore that not all the micelles are, in pure solution, unimolecular and that additional organic substance can either increase or decrease the aggregation.

Amphipathic polymers of a different type are the so-called "block" and "graft" polymers. In the former, blocks of hydrophobic (e.g. polypropylene oxide) and hydrophilic (e.g. polyethylene oxide) units alternate in the main chain. In the latter, the main chain of one type is caused to branch and the branches continued by polymerization of another type of monomer. Despite the small differences in the units, the polymer, as a result of the "exaggeration" effect, has strongly amphipathic properties. This subject is yet in its infancy and its study has not yet contributed to the solution structure which is the subject of this article.

X. Different Liquid Phases

At sufficiently high temperatures and concentrations, a second or even third liquid phase may appear in aqueous solutions of simple paraffin chain salts. In such two-component systems, of course, three liquid phases can only occur at an invariant triple point. The tendency to formation of the higher-concentration phases is, however, generally increased by the presence of other electrolytes, and it is then possible, as in the hot concentrated systems containing sodium chloride which are met with in soap-boiling practice,[47] for three liquid phases to appear together over a range of temperatures. The formation of other phases can also be facilitated by the addition of non-electrolytes and these need not be water-immiscible.

The more concentrated phases can be either isotropic or anisotropic, but three distinct isotropic phases do not seem to be possible.

The existence of partially miscible pairs of liquids is commonplace, but it is only with the organic electrolytes in which one ion at least has a considerable organic portion that we meet this phenomenon with salts in water.

Aggregation of paraffin chain ions occurs at quite low concentrations in water —at about N/100 for C_{12} salts (*ca.* 0·2 per cent) and at about N/1000 for C_{16} salts (*ca.* 0·03 per cent). The dilute phase, therefore, has a peculiar non-random

solution structure long before a concentration is reached at which a second stable phase occurs. This phase, therefore, also has presumably a special structure which clearly cannot be the same.

The most probable structure of the second phase, if also isotropic, is that it involves an aggregation of the inverse type, the phases differing as do oil-in-water and water-in-oil emulsions. It contains a water-expanded form of the type of aggregate found in oil solutions of soaps, but the continuous part of the solution consists entirely of attached chains without added solvent. Naturally the addition of a paraffin- rather than water-soluble solvent facilitates this arrangement at high concentrations. SCHULMAN and RILEY have demonstrated the existence of the two types of aggregation in amicroscopic emulsion systems.[48]

What are the factors which favour the formation of the inverse phase? Firstly, high concentration. A close-packed assembly of equal spheres has $\pi/3\sqrt{2} \simeq 74$ per cent of its volume within the spheres and 26 per cent between them. Allowing for extension of the spherical paraffin chain aggregate by hydration and a range of coulomb force action considerably exceeding the actual radius, we should expect very serious interference of the aggregates on one another at a considerably lower volume concentration of the salt than 74 per cent. The first effect of this interference we have already noted, i.e. to set the spheres, by mutual repulsion, more nearly in a close-packed lattice than in a random distribution, and it is this effect which gives rise to the regular long spacing made evident in x-ray diffraction studies.

At higher concentrations there must occur a deformation of some kind. We have already noted that the preference for a sphere over a prolate spheroid is a rather delicate one, and that in some salts, particularly in the presence of added simple salt, a very long prolate spheroid or indefinite fibril is preferred. Were a higher concentration of paraffin chain salt to be attained by a change from sphere to fibril, the result could be achieved only if the fibrils lay generally parallel one to another. By this means a maximum volume fraction of $\pi/2\sqrt{3} \simeq 91$ per cent could be attained. Such an arrangement, however, would be anisotropic.

In many of the simple salts a volume concentration of more than 74 per cent is reached before the second phase appears, and this may still be isotropic. The fibrillar arrangement seems unlikely, and it is more probable that a more nearly polyhedral type of distortion of spheres occurs until the structure collapses to the inverse one. Although the coulombic forces might be expected to produce distortion at considerably lower concentrations than 74 per cent, their existence is probably in another way responsible for the persistence of the paraffin-in-water structure above its simple geometrical limit. Consider simplified units of the two structures, on the one hand a sphere of paraffin in water and on the other one of water in paraffin. The spheres we will assume of equal size, each carrying a covalently-secured array of electric charges in its surface. The compensating cloud of small oppositely charged ions is in the first case exterior. It is attracted to the sphere by coulomb forces, but this

attraction is lessened by its own charge and by the dispersive effect of thermal energy. In the second case this cloud is interior. To a first approximation the external shell of charge has no influence in the centre, and the free ions are forced outwards by their own charges without any compensation. In so far as, by non-random distribution, the charges in the shell have interior influence, this acts in the same direction as the mutual repulsion of the small ions. The inverse system therefore tends to leave the innermost water unpopulated by ions. It does not therefore offer a satisfactory mechanism for a solution process dominated by the attraction between ions and water molecules. This effect is accentuated because, in an inverse phase in moderate concentrations of paraffin chain salt, the water spheres will be larger than the paraffin spheres in the normal solution structure.

To make this point clear we may introduce here a brief consideration of the geometry of a suspension of mutually repulsive spheres. The arrangement which gives the densest possible packing of equal spheres is of course also the one which, at greater dilution, sets each sphere at the greatest possible distance from its nearest neighbours. In this arrangement, the centres of four spheres in mutual contact are at the corners of a regular tetrahedron and the centre of each sphere is at the centre of a regular eicosahedron at the twelve corners of which are the centres of the twelve equidistant nearest neighbours.

If the centre-to-centre distance of neighbouring spheres is $2l$ and their radius is r, the total volume containing N spheres (where N is large) is $4 \cdot \sqrt{2} \cdot N \cdot l^3$, while the total volume inside spheres is $\frac{4}{3}\pi \cdot N \cdot r^3$.

The volume fraction, ϕ, occupied by spheres is therefore

$$\phi = \frac{\pi}{3\sqrt{2}} \cdot \frac{r^3}{l^3}$$

which has a maximum value of $\pi/3\sqrt{2} \simeq 74$ per cent when the spheres are in contact ($r = l$).

It is from this equation that we may calculate the radius of the spheres from the long spacing, $2l$, obtained from x-ray diffraction measurements and the volume fraction, ϕ. The result, as has been indicated above, gives a reasonably constant value of r and, in agreement with the diffusion measurements, this is about the length of a fully extended chain.

The "inverse" phase is not, however, characterized by constant r, but rather by a maximum distance between the surface of the spheres (now aqueous) and the most remote point between neighbouring spheres, since this space between spheres must be wholly filled by paraffin chains of limited length, L.

The distance from sphere centre (i.e. tetrahedron corner) to the most remote point between spheres (i.e. tetrahedron centre) is $(3/2)^{\frac{1}{2}} \cdot l$ and hence the above condition gives

$$\left(\frac{3}{2}\right)^{\frac{1}{2}} l - r \leqslant L$$

Since r must presumably be maximal, we can substitute the limiting equality above in the volume fraction equation (ϕ being now the volume fraction *of water*) and obtain

$$\frac{L}{r} = \left(\frac{3}{2}\right)^{\frac{1}{3}} \cdot \left(\frac{\pi}{3\sqrt{2}\phi}\right)^{\frac{1}{3}} - 1 = 1{\cdot}224\left(\frac{0{\cdot}74}{\phi}\right)^{\frac{1}{3}} - 1$$

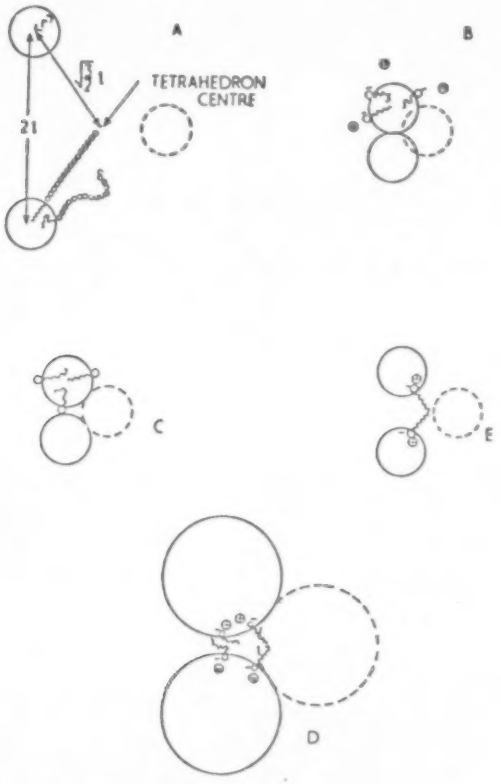

Fig. 4. Scale representation of spherical micelle spacings.

Section through two neighbouring spheres and the centre of a tetrahedron of sphere centres including them. The other pair of spheres are above and below the broken circles. Paraffin chains are represented by zig-zag lines and hydrophilic groups by small circles.

In *A*, *B*, and *C*, we have paraffin spheres of size determined by the necessity for a fully extended chain to reach the centre from the surface.

At *A* $1 = 4r$ and the paraffin occupies only 1·17 per cent of the total volume. Nevertheless a fully extended hydrophilic chain three times as long as the paraffin chain could reach to the tetrahedron centre and so, at a total solute concentration of only 4·7 per cent, interference of the micelles is possible.

With small ionic heads, we have to attain near contact of the spheres (*B*, 74 per cent *v/v*) before interference is necessary.

C represents a 50 per cent *v/v* solution with paraffinic spheres of radius determined by chain length. *D* represents the same solution in the inverse structure where the size of the water spheres is determined by the necessity of fully extended chains to reach the tetrahedron centre. The water spheres cannot become as small as the paraffin spheres in *C* until the paraffin phase (continuous) has reached 87 per cent *v/v* as at *E*.

Structure *D* is improbable with a pure paraffin-chain salt solution because the ions are not able to distribute themselves throughout the large water spheres. It is, however, clearly possible with a non-ionic agent with long hydrophilic chains.

We note that $\frac{L}{r}$ increases, and therefore r decreases, with increasing dilution of water, but the maximum value of r in the most concentrated solution of water in salt (74 per cent v/v) is *ca.* 4·5 × L. r falls to the value L, i.e. the water spheres become as small as the paraffin spheres in the normal type of aqueous solution, when the volume concentration of water falls to *ca.* 17 per cent.

The second phase appears to form much more readily in solutions of the unionized amphipathic substances, and this of course is to be expected on the above reasoning, since coulomb forces have ceased to play an important part. The phase relationships in this type of substance seem only to have been worked out so far in the case of the polyethylene oxide compounds.

We should in general, however, expect a geometrical factor also to be important in the same direction. In the paraffin chain salts the hydrophilic group is small and intensely hydrophilic, whereas in the unionized compounds a weaker hydrophilic tendency is spread over a much larger group. For example, Lissapol N, which has about 12 ethylene oxide groups, condensed on to one octyl phenol molecule, has a hydrophilic chain which, when fully extended, is about three times as long as the hydrophobic one. If we imagine that at least some of these hydrophilic chains can extend outwards radially from the surface of the central hydrophobic sphere, we realize that the spherical aggregates can undergo direct interference with one another when these extended hydrophilic groups touch. Now the volume of this outer sphere of influence is $(4^3 - 1^3) = 63$ times as great as the central hydrophobic volume, although the volume of solvent therein of course is only three times as great. Consequently mechanical interference becomes inevitable at a volume concentration of the solute equal to $\frac{4}{64} \times 74$ per cent = 4·67 per cent. It may easily happen therefore that at a concentration not much above 5 per cent the inverse structure would be more convenient. Without distortion of spheres, the minimum volume concentration of hydrophobic material in such a structure is 26 per cent, which is approximately the hydrophobic fraction in the amphipath alone, so that, to include water at all, some distortion or solution of polyethylene oxide in the spheres is necessary.

Lissapol N in water, at temperatures below 28°C, forms only one phase (isotropic) until a concentration of 46 per cent is reached.[44], [49] Above 79 per cent the solution is also single phase and isotropic. Between these concentrations the solution divides into an anisotropic phase and one or other of the isotropic ones.

Above 28°C separation of dilute solutions into two isotropic phases occurs, one of which is of low concentration but still above the micelle concentration (0·2 per cent), and an anisotropic phase occurs now at a lower concentration than 46 per cent. The two dilute isotropic phases are probably of the paraffin-sphere and water-sphere type. The concentrated isotropic phase probably contains its water as attached water of hydration of the ether oxygen atoms. Whether the isotropic phase contains filament micelles or lamellae is not clear.

The tendency in this case for separation to occur at higher temperatures is doubtless connected with the same phenomenon in solutions of pure polyethylene oxides. The water-ether association presumably is more quickly loosened by increase of temperature than water-water association.

Since the ionic dissociation of the electrolyte in water is a factor tending to discourage the formation of the inverse phase, it is not surprising that these phases form more easily when the neutralizing ion is divalent than when it is univalent, because in the former case there is a much greater tendency to ion-pair formation, whatever the structure of the solution. For a related reason, the addition of simple salts to the paraffin chain salt solution has an even greater effect in facilitating the formation of the inverse phase, because the interior of each isolated region of water can now contain small ions of both signs, which are not therefore mutually repelled to the periphery. When both effects are combined, the inverse phase forms very easily. The writer, for instance, has found that a 1 per cent aqueous solution of the copper salt of the sulphonate of cetyl *para*tolyl ether at 80°C forms globules of an inverse phase containing most of the paraffin chain salt on the addition of a small concentration of copper sulphate. In this case, and probably in many others, where phase separation occurs at a very low total concentration of amphipathic substance we are probably not, however, dealing with phases having normal and inverse types of aggregation.

We have so far assumed that, since aggregation occurs in very low concentration in aqueous solutions, the more concentrated phase must have an equally specialized structure. We should, however, bear in mind that there are numerous examples of pairs of partially miscible liquids in which no very specialized structure is present. In fact, liquids are only completely miscible when the discrepancy between the inter-molecular attractions is not very great. Where the discrepancy is great, a single molecule of one component present amongst molecules of the other is restrained by much less powerful forces than a single molecule present amongst its own kind. Consequently the escaping tendency of a substance may be the same at two very different concentrations. In one case we have a high concentration and a low possibility of any individual molecule escaping; in the other we have a low concentration but a much higher possibility of any individual molecule escaping. We are not normally accustomed to thinking of any such generalized type of partial miscibility occurring in the case of electrolytes in water, because the ordinary electrolytes of common experience are crystalline solids of high melting point where the crystal forces are the main factor limiting solubility, the ions, when free, having unambiguously strong attraction for water molecules. If we have a molecule containing a mixture of groups which are attractive to and groups which are not attractive to potential solvent, the normal effect is an averaging of these tendencies, and therefore a partial miscibility. It is only because the attractive and unattractive groups in the case of the paraffin chain salts are as far as possible separate that the peculiar structure of these solutions is possible. If we could have an electrolyte containing hydrophobic and ionic groups more

intimately mixed and also incapable of forming a crystal, we might expect to find the normal type of partial miscibility, even in an electrolyte in water.

The aggregation of the paraffin chain salts causes a much reduced rate of change with concentration of the thermodynamic properties of both components when the critical concentration is exceeded, and therefore a dilute solution just below the critical concentration is not so very far from being in equilibrium with a much more concentrated solution above the critical concentration. A comparatively minor change of conditions might therefore cause a separation into two phases, one of which would now be a very dilute solution containing only molecularly dispersed solute, while the more concentrated solution would have the structure, which we have described above, of the normal aqueous solution. Where a second phase results on the addition of some simple electrolyte, in comparatively low concentration of the paraffin chain salt, it is probable that we are dealing with a partial miscibility of this type, the dilute phase being very dilute in paraffin chains and containing them only in the unaggregated state, and the concentrated phase having a normal paraffinic aggregate structure with a lower concentration of the added electrolyte in the inter-micellar liquid.

Naturally the tendency for this to occur will be greater where the normal type of aggregation is difficult to accomplish, i.e. in solutions of salts having double or branched chains, and this view is consistent with the very early occurrence in this system of a second liquid phase. To the writer's knowledge it is possible to realize two phases in the system, water—simple salt—organic salt, when the organic salt is one, such as ammonium phenoxyacetate, which we do not normally consider to be aggregated. It is in fact easier to see why separation should occur when the organic ions are not aggregated, because their presence in true solution will contribute the effect of an organic diluent and so make the water a less good solvent for the simple salt. This is necessary because the phase poorer in organic salt must contain a higher concentration of simple salt to reduce the thermodynamic potential of the water to that in the organic-rich phase.

We have dealt above with the possible structures of the isotropic phases in equilibrium with one another. Concentrated phases of an anisotropic character can also occur. It is clear that these must have a very different structure. The anhydrous salts are frequently found to melt, where they are sufficiently stable to withstand the necessary temperatures, to anisotropic liquids before they become isotropic at a second melting point. These liquid crystalline forms are in the smectic state, where they have much greater fluidity in two cartesian directions than in the third, which is generally considered parallel to the paraffin chains. Under the microscope they show very clearly in thin films the stepped drop phenomenon of GRANDJEAN.

The normal paraffin chain salt crystals consist of alternate paraffinic and ionic layers, the chains in the former lying parallel to one another and inclined steeply to the planes. It is entirely to be expected that fusion to a limited extent may occur in the paraffinic layers, the chains becoming free to rotate

about their axes and therefore to move about one another in directions perpendicular to their axes before, at a higher temperature, they enter into the completely fluid state. In the smectic state therefore the molecular layers can be stirred within themselves, but are only with much more difficulty mixed together. The extremely polar nature of the ionic lattice layers between the paraffin layers is the chief factor in restricting the fluidity in the third direction. Its rigidity tends to resist the natural stirring within the planes. It is entirely to be expected that a small amount of water expanding and dissolving the ionic lattice will greatly increase the tendency to formation of a smectic liquid, which then appears at a lower temperature as a new phase of concentrated solution.

The high concentration smectic state has therefore the lamellar-micelle structure. It is here that the expected properties of the lamellar-micelle are to be found—indefinite aggregation, and anisotropy, both rheological and optical. The unquestionable existence of this structure in the anisotropic high concentration phase is in itself another argument against its existence in an entirely different and more dilute phase which may co-exist with it.

We have said above that more than one anisotropic phase can exist. There has been inadequate study of this subject to enable one to say what are really the essential differences between them. Since the liquid crystalline phase is in part crystalline, it is of course possible for various stages of fluidity to arise at different temperatures, in much the same way as transitions from one crystalline form to another. The fatty acids themselves, for instance, can exist in more than one solid crystalline form, and the differences between them are mainly due to differences in inclination of the paraffin chains to the planes of the layers. It is clearly possible for two smectic phases to differ in this respect.

We have also seen that, under some conditions, an indefinitely elongated rod-like assembly of paraffin chain ions is preferred, and that this arrangement is definitely anisotropic. It is possible, therefore, and indeed probable, that one of the anisotropic phases to be met with in equilibrium with isotropic ones is a concentrated solution of this structure.

XI. Solutions of Amphipathic Substances in Non-polar Solvents

We have seen that some of the paraffin chain salts can dissolve in non-polar solvents. Boiling point or freezing point measurements have clearly shown that the molecular weight of the dissolved substance indicates a fairly high degree of aggregation.[59] This aggregation is obviously the inverse type to that found in dilute aqueous solutions. The ionic centre of the aggregate is presumably in a crystalline condition and the limitation on size, which is probably not so definite as in the aqueous systems, is caused by the necessity for the outstanding paraffin chains not to be in parallel crystalline alignment. If they were, then of course the growth of the crystal could not be prevented, and in fact the substance would be above its limits of solubility.

That the interior of the micelle is in this sense rigid is suggested by the successful application of an ingenious method for the determination of molecular weight. In this method a fluorescent dye is absorbed on the micelle, and

measurement of depolarization of the emitted light is made.[51] Were the absorbed dye perfectly free to rotate as a molecule, almost complete depolarization would result, but it appears that it is rigidly incorporated in the micelle, so that the depolarization measurement indicates the amount of revolution of the micelle which occurs during the short lifetime of the excited molecule. Since very good agreement is obtained between the results of this method and direct osmotic pressure measurements, it is clear that the theory is well-founded.

Many solutions of amphipathic substances in non-polar solvents can show similar solvent properties to that of the aqueous solution. That is to say that polar solvents, particularly water, can be taken into solution to a much higher concentration than in the solvent alone. The amount of water which can be taken up is very dependent on the salt chosen and this is not surprising in view of the different degrees of hydration of different ions and the importance of geometrical factors in the stability of the crystal nucleus, which is presumably lost during the solution process. The solvent-power for water of the dissolved paraffin chain salt also depends on the main solvent[52] and it is of interest that it is higher with aliphatic hydrocarbons, intermediate with benzene, and lowest with the more polar chlorinated hydrocarbons, such as chloroform and methylene chloride.

The addition of water to these solutions does not always result in a greater mutual solubility.[53] The calcium salts, for instance, are much more soluble in hydrocarbons when dry. This is doubtless a complication introduced by the different crystal structures of hydrated and unhydrated salts.

These solutions in non-polar solvents are of course basically similar in structure to the inverse aggregation proposed as a possibility for the second isotropic phase which can occur in concentrated aqueous solutions.

XII. Solutions in Polar Solvents and Mixtures

We have seen that the lower alcohols form an intermediate type of solvent for paraffin chain salts, in which neither type of aggregation appears to occur to any considerable extent. Consequently the solvent power of the lower alcohols is very general, but never very great.

In general the higher alcohols, such as amyl alcohol, cyclohexanol and phenol, are very good solvents for the paraffin chain salts and give mobile isotropic solutions, the structure of which does not seem to have been seriously investigated. Solutions in cyclohexanol particularly have frequently been used as liquid soap preparations.

The addition of water to solutions of paraffin chain salts in these solvents almost invariably results in a very great increase of viscosity and the development of anisotropic behaviour, although when water is added in large excess the whole system is dissolved again to a mobile isotropic solution, in which presumably the higher alcohol is incorporated in the normal type of micelle. Addition of suitable amphipathic co-solvents, such as hexanol and octanol, greatly extends the range of concentration of paraffin chain salt over which

anisotropic structure persists. No firm evidence has been found of the structure of these intermediate anisotropic phases. WINSOR[54] considers that we have, so to speak, presented the paraffin chain salt with so indefinite a choice between aggregation with paraffin chains inside and aggregation with paraffin chains outside, that the only answer is an indefinite lamellar aggregation with the paraffin chains parallel. This may well be the case, but we must again bear in mind that an assembly of long rod-like aggregates would also be anisotropic.

WINSOR's view is supported by the following considerations.

We have seen that the solution of a liquid hydrocarbon in paraffin chain salt solutions is not very great, and is due to its incorporation in the inside of a liquid micelle without any serious change in its properties except a swelling, which necessitates the limitation of solubility because the innermost portion of the swollen micelle contains the added hydrocarbon only.

If we add, however, a highly polar liquid which is itself amphipathic, such as a higher alcohol, the solubility in the micelle is generally much greater, and this is doubtless due to the fact that the added solvent itself is oriented in a similar way to the paraffin chain. Being, however, also paraffin-soluble, it can expand the interior of the micelle as well. Expansion may therefore reach a stage where the diluted layer of paraffin chain ions in the surface has so little curvature that little further energy change is involved in its becoming flat.

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3

APPLICATIONS OF LOW TEMPERATURE CRYSTALLIZATION IN THE SEPARATION OF THE FATTY ACIDS AND THEIR COMPOUNDS

J. B. Brown and Doris K. Kolb

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3

APPLICATIONS OF LOW TEMPERATURE CRYSTALLIZATION IN THE SEPARATION OF THE FATTY ACIDS AND THEIR COMPOUNDS

*J. B. Brown and Doris K. Kolb**

I. Introduction and Historical

Historically, the chemistry of the fats may be divided for convenience into three periods. (1) *The Classic Period* ended in 1921, the year of the appearance of the last edition of Lewkowitsch's famous treatise on fats, oils, and waxes (Lewkowitsch, 1921). Up to this time, there had accumulated a vast amount of semi-quantitative data on the fats and on the nature of their component fatty acids. (2) *The Transitional Period* of fat chemistry, 1921–35, was characterized by numerous attempts to improve the classic methods for the study of the composition of the fatty acid mixture, and by the beginning and partial fruition of Hilditch's noteworthy contributions to our knowledge of glyceride structure. (3) *The Modern Period*, 1935 to date, has been attended by the development of new and more efficient procedures for separating and studying fatty acid mixtures and naturally occurring lipid mixtures so that our knowledge of the nature and composition of the natural fats and oils is year by year becoming more exact.

These new procedures for the separation of lipids, and especially their component acids, are the following:

(*a*) *Precise ester distillation*

Methyl ester distillation had been used since 1906 for the partial separation of fatty acid mixtures (Haller, 1906). Although the apparatus then available for this purpose was inefficient, the fact that the fatty acids usually occur naturally in series differing by two carbon atoms made it possible by the use of repeated through-puts to achieve fair separations. The development of efficient packed columns has made it possible to almost completely separate these carbon series in one through-put (Schoenheimer and Rittenberg, 1937; Longenecker, 1937; Todd, 1945; Norris and Terry, 1945; Murray, 1951). By very careful work, it is even possible to separate methyl oleate and methyl stearate, the boiling points of which differ by only 2°C at pressures ordinarily used for their distillation. On the other hand, because of association in the vapour phase, separation of methyl oleate and linoleate is not possible by this method.

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(b) Chromatographic adsorption

This procedure has been highly developed in a number of laboratories and has been recently reviewed by HOLMAN in Volume 1 of this series (1952). By the use of appropriate adsorbents, it is possible to separate fatty acids (or their esters) according either to carbon series or to degree of unsaturation and also by virtue of other factors in the chemical structure of the acids in question. Chromatographic adsorption has made it possible to isolate the important essential fatty acids, linoleic, linolenic, and arachidonic, by purely physical methods without the necessity of employing the questionable bromination-debromination procedure.

(c) Countercurrent extraction

This procedure has been developed mainly by CRAIG and his associates at the Rockefeller Institute for Medical Research (CRAIG *et al.*, 1947; CRAIG and POST, 1949; CRAIG and CRAIG, 1950; CANNON *et al.*, 1952). The subject is reviewed in Volume 2 of this series (DUTTON, 1954). The procedure is based on differences in distribution of fatty acids (or esters) by molecular weight and/or unsaturation between two partially miscible solvents. It is possible by this method to separate the higher saturated acids, as well as mixtures of oleic, linoleic and linolenic acids.

(d) Crystallization of urea complexes

Urea forms definite complexes with the fatty acids, and it has been possible to effect workable separations based on differences in solubilities of these complexes in various solvents (SCHLENK and HOLMAN, 1951; SWERN and PARKER, 1952, 1953). A review of this subject appears in Volume 2 in the present series (SCHLENK, 1954).

(e) Low temperature crystallization

This procedure, developed originally in this laboratory, has been useful in the separation of glycerides, fatty acids, esters, and other lipids, and particularly for the separation of those types of components which are highly soluble in organic solvents at temperatures above 0°C but which become sparingly soluble at temperatures down to − 80°. It is the purpose of this chapter to review the applications of this method to lipid chemistry.

Previous to 1937, procedures for the separation of fatty acid mixtures were mainly of two types—relatively crude ester distillation for the separation of acids by carbon series and metal salt separations based on differential solubilities of soaps in various solvents. The latter type is exemplified by the lead soap-ether or alcohol separations of solid and liquid, saturated and unsaturated, acids, procedures which are still official for the American Oil Chemists' Society and the Association of Official Agricultural Chemists.

Direct crystallization of fatty acids from solvents had previously been used mainly to purify individual saturated acids and to effect crude separations of saturated and unsaturated acids. In working with unsaturated acids, its use

was limited by their high solubilities in the organic solvents at the temperatures commonly available.

In 1937 two papers (BROWN and STONER; BROWN and SHINOWARA) were published from this laboratory describing the partial separation of the fatty acids of cottonseed oil and the preparation of oleic acid from olive oil by low temperature crystallization; in this work advantage was taken of the wide solubility differences in solvents at low temperatures between saturated, oleic, and linoleic acids. Although oleic acid had been partially purified before 1937 by crystallization at temperatures below 0°, this was the first time that temperatures as low as — 75° had been employed. The development of low temperature crystallization methods for the investigation of lipids has been made possible in large part by the wide commercial availability of dry ice. In those laboratories where this commodity is not yet available, mechanical equipment for developing low temperatures can now be substituted for dry ice. Applications of the method have expanded rapidly. Work up to early in 1941 was reviewed by BROWN (1941); the present chapter includes work done mainly since that time.

Since the crystallization method is based primarily on solubility differences between various fatty acids or their esters, including glycerides, typical solubility data will be first presented, and this will be followed by a discussion of certain practical considerations limiting application of these data. A variety of applications of the method in the separation of fatty acids and their compounds will then be cited. A limited number of examples in organic chemistry will also be described in the belief that the method can be made widely useful in the separation of low melting or liquid organic compounds.

II. Solubilities of the Fatty Acids (and Esters) in Organic Solvents

The separation of fatty acid mixtures by crystallization from solvent depends first of all on solubility differences between the various components of the mixture. Although it has long been known that the higher saturated acids are much less soluble than the corresponding unsaturated acids, and advantage has often been taken of this fact to partially separate mixtures, few, if any, accurate solubility data on pure fatty acids had been reported previous to 1941. Such data as had been reported were obtained on specimens of acids of questionable purity. No accurate data were available for the unsaturated acids.

It has been emphasized by BAILEY (1950) and others that the solubility of any given acid is closely related to its melting point, and is to a certain degree dependent on the nature of the solvent. In general, the even carbon, normal saturated and unsaturated acids become less soluble with increase in molecular weight; it may be further stated for the unsaturated acids that solubility increases with the number of double bonds. Also, the *trans* acid of a *cis-trans* pair (higher melting) is less soluble than the *cis* acid (lower melting).

During the early work in our laboratory, we realized that fundamental solubility data on the fatty acids and their compounds were essential to the full

development of the technique of low temperature crystallization. The major usefulness of the technique is as a tool for the separation of saturated and unsaturated acids and of mixtures of the important unsaturated acids. Therefore, work was begun in 1940 on the determination of solubilities of a series of saturated and unsaturated acids in methanol, acetone, and Skellysolve-B at temperatures from 0° to — 70°. This work was reported in 1944 (FOREMAN and BROWN). In the meanwhile, RALSTON and HOERR (1942; 1944) had published two papers describing the solubilities of even and odd carbon acids from C_8 to C_{18} inclusive in a large series of solvents. Their data on the odd carbon acids, while of great fundamental importance, have little significance in the separation of naturally occurring mixtures. Since most of the natural lipids contain no saturated acids below C_{12}, we have summarized this early work by RALSTON's group in Table 1 which lists the solubilities of lauric, myristic, palmitic, and stearic acids at 0°. Later data by HOERR *et al.* (1946) included solubilities in toluene, o-xylene, chlorobenzene, furfural and several other solvents. In Tables 2, 3, and 4, we are reporting in full the data by FOREMAN and BROWN.

Table 1. Typical solubility data on saturated acids
(RALSTON and HOERR, 1942, 1944)

Solvent	*Solubility at 0°, grams per 100 gm solvent*			
	Lauric	*Myristic*	*Palmitic*	*Stearic*
Ethanol (99·4%)	20·4	7·07	1·89	0·42
Ethanol (95·0%)	15·2	3·86	0·85	0·24
Ethanol (91·1%)	—	—	0·76	0·13
Ethanol (80·8%)	—	—	*ca.* 0·06	—
Acetone	8·95	2·75	0·60	0·21
2-Butanone	11·5	4·28	0·90	0·25
Benzene	32·3[a]	6·95[a]	1·04[a]	0·24[a]
Glacial acetic	81·8[b]	10·2[b]	2·14[b]	0·12[b]
Cyclohexane	19·8[a]	5·3[a]	0·9[a]	0·2[a]
Carbon tetrachloride	9·2	3·2	0·6	—
Chloroform	22·4	8·1	2·9	0·4
Ethyl acetate	9·4	3·4	0·8	—
Butyl acetate	13·0	4·8	1·5	< 0·1
Methanol	12·7	2·8	0·8	—
Isopropanol	21·5	7·2	2·4	< 0·1
n-Butanol	21·4	7·3	1·9	—
Nitroethane	1·9	0·3	—	—
Acetonitrile	2·1	0·7	< 0·1	—

[a] Solubility at 10°. [b] Solubility at 20°.

The data in the preceding tables explain fully the successful separations of fatty acid mixtures, reported before solubilities of their components were known. Thus, at — 20° oleic acid is soluble in all proportions in methanol, while stearic acid is soluble only to the extent of 0·01 gm per 100 gm of solution. In a number of other solvents, oleic acid is 14·7 to 500 times as soluble as palmitic acid. In acetone at — 70°, linoleic acid is thirteen times as soluble as oleic, while, in turn, linolenic is about four times as soluble as linoleic acid.

Table 2. Solubilities of the saturated acids

(FOREMAN and BROWN, 1944)

Solvent Temp. °C	*Acetone grams/1000 g sol'n.*	*Methyl alcohol grams/1000 g sol'n.*	*Skellysolve B grams/1000 g sol'n.*
		Lauric acid	
0°	—	—	15·1
− 10°	—	28·3	10·7
− 20°	17·4	17·0	3·79
− 30°	12·3	8·23	—
− 50°	0·25	—	—
		Myristic acid	
0°	22·7	18·4	9·43
− 10°	10·7	8·26	3·25
− 20°	4·33	3·44	1·31
− 30°	1·74	1·53	0·20
− 50°	—	—	—
		Palmitic acid	
10°	17·7	13·1	8·38
0°	7·15	3·96	1·25
− 10°	2·80	1·46	0·26
− 20°	1·34	0·63	0·12
− 30°	0·48	0·20	0·09
		Stearic acid	
10°	4·69	2·59	1·31
0°	2·19	0·92	0·24
− 10°	0·38	0·32	0·10
− 20°	—	0·10	—
		Arachidic acid	
10°	1·83	0·96	0·56
0°	0·75	0·65	0·48
		Behenic acid	
10°	0·51	0·42	0·29
0°	0·10	0·10	0·10

Table 3. Solubilities of the unsaturated acids
(FOREMAN and BROWN, 1944)

Solvent Temp. °C	*Acetone grams/1000 g sol'n.*	*Methyl alcohol grams/1000 g sol'n.*	*Skellysolve B grams/1000 g sol'n.*
		Eicosenoic acid	
− 20°	16·1	10·6	—
− 30°	4·58	3·48	2·95
− 40°	1·47	1·29	1·07
		Erucic acid	
− 20°	3·52	1·76	—
− 30°	—	0·87	0·59
		Oleic acid	
− 30°	14·2	7·08	11·8
− 40°	5·16	3·29	4·83
− 50°	1·89	0·89	1·04
− 60°	0·61	0·51	0·42
− 70°	0·40	0·32	0·24
		Linoleic acid	
− 50°	48·2	25·2	17·0
− 60°	14·2	9·25	3·27
− 70°	5·19	3·94	0·09
		Linolenic acid	
− 62°	43·2	17·6	4·43

*Table 4. Solubility ratios of fatty acids under various conditions**
(FOREMAN and BROWN, 1944)

Solvent	*Temperature*	*Oleic*	*Palmitic*	*Ratio Oleic : Palmitic*
Methyl acetate	− 25°	10·0	0·74	14·7 : 1
Acetone	− 30°	14·2	0·48	30·0 : 1
Methyl alcohol	− 30°	7·08	0·20	35·4 : 1
Butyl alcohol	− 25°	62·8	1·32	47·6 : 1
Ethylidene dichloride	− 25°	26·8	3·24	82·7 : 1
Skellysolve B	− 30°	11·8	0·09	130·0 : 1
Carbon disulphide	− 30°	15·7	< 0·1	> 157·0 : 1
Toluene	− 30°	50·2	< 0·1	> 500·0 : 1
Ethyl ether	− 40°	43·7	< 0·1	> 450·0 : 1

* Solubilities expressed gm per 1000 gm solution. Numerous other ratios can be derived from the preceding tables.

Table 4 (continued)

Solvent	*Temperature*	*Linoleic*	*Oleic*	*Ratio Linoleic : Oleic*
Skellysolve B	− 70°	0·60	0·24	2·5 : 1
Carbon disulphide	− 62°	4·12	0·398	10·3 : 1
Methyl alcohol	− 70°	3·94	0·32	12·3 : 1
Acetone	− 70°	5·19	0·40	13·0 : 1

In addition to the work reported above, the Armour group have since published extensive solubility data on a series of compounds related to the fatty acids or to their derivatives such as fatty alcohols (HOERR *et al.*, 1944a), ketones (GARLAND *et al.*, 1943), primary amines (RALSTON *et al.*, 1944a), secondary amines (HOERR *et al.*, 1944b), tertiary amines (RALSTON *et al.*, 1944b), long chain amides, anilides and substituted amides (RALSTON *et al.*, 1943), primary amine hydrochlorides (SEDGWICK *et al.*, 1945), alkyl quaternary ammonium salts (EGGENBERGER *et al.*, 1950), dialkyl dimethyl ammonium chlorides (RALSTON *et al.*, 1948), and nitriles (HOERR *et al.*, 1944c).

In a more recent study of the solubility behaviour of esters of saturated acids, SEDGWICK, HOERR, and HARWOOD (1952) concluded that the property of association in solution, generally shown by the saturated acids, is not shown by the esters. Unfortunately, practical advantage cannot yet be taken of this finding, because no data on unsaturated esters are included in their work. Some of their results on esters follow in Table 5.

Table 5. Solubilities of alkyl esters in various solvents
(SEDGWICK, HOERR, and HARWOOD, 1952)*

Ester	*Grams of ester per 100 gm of solvent*								
	− 50·0°	− 40·0°	− 30·0°	− 20·0°	− 10·0°	0·0°	10·0°	20·0°	30·0°
Chloroform									
Me caprylate	120	375	∞	∞	∞	∞	∞	∞	∞
Me laurate	25·0	33·3	48	77	140	440	∞	∞	∞
Me myristate	10·2	12·8	18·3	29·2	51	103	285	∞	∞
Me palmitate	1·8	3·1	6·4	12·3	24·0	50	106	290	∞
Me stearate	< 0·1	0·3	1·9	5·9	13·6	28	54	120	360
Ethyl acetate									
Me caprylate	76	340	∞	∞	∞	∞	∞	∞	∞
Me laurate	0·4	1·9	5·4	20·0	80	460	∞	∞	∞
Me myristate	—	0·3	1·2	4·1	12·4	40	225	∞	∞
Me palmitate	—	—	0·3	1·0	2·7	8·6	34·8	215	∞
Me stearate	—	—	—	0·1	0·5	2·1	8·6	42	350
Et stearate	—	—	—	0·3	1·0	3·1	14·0	87	1050
n-Pr stearate	—	—	—	0·6	1·5	3·9	21·9	160	∞
n-Bu stearate	—	—	—	0·9	1·8	4·3	27·2	250	∞

* Data are also given for benzene, cyclohexane, carbon tetrachloride, butyl acetate, *n*-butanol and acetonitrile.

Table 5 (continued)

Ester	Grams of ester per 100 gm of solent								
	− 50·0°	− 40·0°	− 30·0°	− 20·0°	− 10·0°	0·0°	10·0°	20·0°	30·0°
Acetone									
Me caprylate	64	380	∞	∞	∞	∞	∞	∞	∞
Me laurate	0·2	0·9	3·6	12·5	67	645	∞	∞	∞
Me myristate	—	—	0·5	2·1	7·1	28·7	248	∞	∞
Me palmitate	—	—	0·1	0·4	1·3	5·2	21·0	250	∞
Me stearate	—	—	—	—	0·1	0·7	3·6	19·9	450
Et stearate	—	—	—	—	0·4	1·5	5·2	40·5	1160
n-Pr stearate	—	—	—	—	0·9	2·0	6·0	185	∞
n-Bu stearate	—	—	—	—	1·2	2·4	6·7	345	∞
Methanol									
Me caprylate	4·2	78	∞	∞	∞	∞	∞	∞	∞
Me laurate	—	—	0·1	1·3	5·3	80	∞	∞	∞
Me tridecanoate	—	—	—	0·3	2·9	36·5	∞	∞	∞
Me myristate	—	—	—	—	< 0·1	1·9	25·0	∞	∞
Me palmitate	—	—	—	—	—	0·1	1·4	27·0	∞
Me stearate[a]	—	—	—	—	—	—	—	0·6	7·2
95·0% Ethanol									
Me caprylate	7·5	57	∞	∞	∞	∞	∞	∞	∞
Me laurate	< 0·1	0·3	1·1	3·8	13·2	120	∞	∞	∞
Me tridecanoate	—	< 0·1	0·5	2·7	11·2	70	∞	∞	∞
Me myristate	—	—	—	0·3	2·0	6·7	30·8	∞	∞
Me palmitate	—	—	—	—	0·2	1·1	5·0	39·4	∞
Me stearate	—	—	—	—	—	0·1	1·2	4·4	40
Et stearate	—	—	—	—	0·2	0·7	2·1	6·6	180
n-Pr stearate	—	—	—	—	0·6	1·5	3·4	11·5	∞
n-Bu stearate	—	—	—	—	1·0	2·1	4·2	18·2	∞

[a] Miscible above 40·8°.

Methyl esters are more soluble in a given solvent than their respective acids. Further, in the series of esters from methyl to n-butyl, solubilities increase with length of the alcohol chain. Thus, in acetone at − 10° butyl stearate is twelve times as soluble as methyl stearate, at 0° 3·3 times, and at 10° two times. Curiously enough at 20°, simultaneous with an enormous increase in solubility, the difference increases to seventeen-fold.

Still more recently, HOERR and HARWOOD (1952) have reported the solubilities of oleic and linoleic acids in a large series of solvents. Their data are included in full in Tables 6 and 7.

In discussing their data, HOERR and HARWOOD comment as follows: "The solubilities of oleic acid in Table 6 refer to those of the form most stable in each

Table 6. Solubility of oleic acid in various organic solvents
(HOERR and HARWOOD, 1952)

Solvent	*Grams oleic acid per 100 gm solvent*						
	− 40·0°	− 30·0°	− 20·0°	−10·0°	0·0°	10·0°	20·0°
n-Hexane	0·1	1·2	9·1	44·4	160	720	∞
Benzene[a]	Eutectic	system	—	—	253	910	∞
Cyclohexane[b]	Eutectic	system	—	80	233	870	∞
Carbon tetrachloride[c]	Eutectic	—	24·6	68	160	590	∞
Chloroform	11·5	23·3	46·0	92	205	760	∞
o-Xylene[d]	Eutectic	—	30·5	88	250	1100	∞
Diethyl ether	1·2	4·4	17·9	60	195	870	∞
Chlorobenzene	2·5	6·2	27·0	85	220	900	∞
1,2-Dichloroethane	—	< 0·1	1·3	26·1	130	670	∞
Nitrobenzene[e]	Eutectic	system	—	—	220	1100	∞
Dioxane[f]	Eutectic	system	—	—	237	1120	∞
Furfural[g]	—	0·1	0·3	1·3	4·7	14·5	27·0
Ethyl acetate	1·6	4·5	12·2	44·0	185	750	∞
n-Butyl acetate	2·8	6·4	14·8	48·0	200	770	∞
Acetone	0·5	1·4	5·1	27·4	159	870	∞
2-Butanone	1·0	2·6	8·6	33·5	170	880	∞
Methanol	0·3	0·9	4·0	31·6	250	1820	∞
95·0% ethanol	0·7	2·2	9·5	47·5	235	1470	∞
2-Propanol	1·1	3·2	11·5	55·0	226	1160	∞
n-Butanol	1·3	4·0	15·2	56·5	100	950	∞
Nitromethane[h]	0·1	0·2	0·4	0·6	0·8	1·0	1·1
Nitroethane[i]	0·2	0·8	1·3	2·2	3·4	8·7	14·3
Acetonitrile[j]	0·1	0·3	0·7	1·1	1·8	7·7	9·1

[a] Eutectic at 59·7 wt per cent oleic acid, − 9·2°. [b] Eutectic at 38·9 wt per cent oleic acid, −12·1°. [c] Eutectic at 9·4 wt per cent oleic acid, − 25·6°. [d] Eutectic at 6·0 wt per cent oleic acid, − 31·0°. [e] Eutectic at 58·5 wt per cent oleic acid, 0·0°. [f] Eutectic at 61·6 wt per cent oleic acid, − 3·3°. [g] Miscible above 26·2°. [h] Miscible above 94·5°. [i] Miscible above 31·7°. [j] Miscible above 61·0°.

of the systems. As would be expected from a dimorphic material, oleic acid exhibits two solubility curves in some solvents. . . . Upon cooling such a system, the solute precipitates as a transparent, unstable, crystalline form, which if the temperature is raised rapidly redissolves at the temperature at which precipitation occurred. If, however, the precipitate is held for an interval of time at or below the precipitation temperature, the material transforms to a more opaque, stable crystalline form which, upon heating, dissolves at temperatures somewhat above those at which precipitation occurred."

The closed tube method which the Armour group have used for their determinations permits solubility measurements for the two forms, but values presented are for the more stable forms. In the method we have used in our laboratory, the system is kept for many hours at a given temperature, so that the values reported are for the higher melting modification.

HOERR and HARWOOD further discuss their results as follows: "Hydrogen bonding between solvent and solute molecules is evidenced by the fact that many of the solubilities of oleic and linoleic acids are greater than would be anticipated on the basis of differences in internal pressures and polarities alone.

Table 7. Solubility of linoleic acid in various organic solvents
(HOERR and HARWOOD, 1952)

Solvent	*Grams linoleic acid per 100 gm solvent*							
	− 50·0°	− 40·0°	− 30·0°	− 20·0°	− 10·0°	0·0°	10·0°	20·0°
n-Hexane	3·0	14·3	53	170	990	∞	∞	∞
Benzene[a]	Eutectic	system	—	320	1250	∞	∞	∞
Cyclohexane[b]	Eutectic	system	—	275	1210	∞	∞	∞
Carbon tetrachloride[c]	Eutectic	—	70	160	600	∞	∞	∞
Chloroform	19·0	40·0	88	210	770	∞	∞	∞
Ethyl acetate	5·6	14·7	58	200	1300	∞	∞	∞
Acetone	3·3	8·6	27·2	147	1200	∞	∞	∞
2-Butanone	4·6	10·6	37·0	185	1220	∞	∞	∞
Methanol	3·3	9·9	48·1	233	1850	∞	∞	∞
95·0% ethanol	4·5	11·1	42·5	208	1150	∞	∞	∞
2-Propanol	6·0	11·7	45·2	203	1080	∞	∞	∞
n-Butanol	8·0	18·9	56	180	870	∞	∞	∞
Nitroethane[d]	—	< 0·1	0·4	2·1	8·3	34·2	∞	∞
Acetonitrile[e]	—	< 0·1	0·2	0·4	4·9	7·5	11·2	17·7

[a] Eutectic at 74·6 wt per cent linoleic acid, − 21·2°. [b] Eutectic at 51·8 wt per cent linoleic acid, − 28·3°. [c] Eutectic at 31·9 wt per cent linoleic acid, − 35·3°. [d] Miscible above 1·5°. [e] Miscible above 39·5°.

Despite the well-known tendencies of the lower alcohols to form strong hydrogen-bonded networks of molecules in their liquid state, the carboxyl hydrogen atom of the unsaturated acids is evidently sufficiently active to compete with the bonding tendencies of the hydroxyl groups of the alcohols. The solubilities of these acids in methanol and ethanol, for example, are considerably greater than they theoretically should be in solvents possessing such high internal pressures. From the limited solubilities in the nitro-paraffins and in acetonitrile, on the other hand, it appears that the carboxyl hydrogen of oleic and linoleic acids is insufficiently potent to disrupt the highly polar fields of nitrogen-containing solvent molecules. Hydrogen bonding characteristics of stearic acid are almost identical with those of these unsaturated acids. The solubility of the fatty acids in chloroform is unique. In this solvent, the unsaturated acids, as well as all the high molecular weight saturated acids, exhibit apparently ideal solutions over wide ranges of concentration."

At the time the preceding work was published, a continuation of our previous solubility investigations was in progress. In this work KOLB (1953) reported further determinations of the solubilities of several of the common higher fatty acids, as well as of several *cis trans* pairs of unsaturated acids, and of one acetylenic acid. Solvents included methanol, acetone, diethyl ether, ethyl acetate, toluene, and n-heptane. Some data were included in which solubilities in a series of hydrocarbons were compared. All of these results were obtained by an improved procedure for securing saturated solutions at given temperatures. Thus, one criticism of our previous work, i.e. the fact that equilibrium was not always attained, has been met by measuring solubilities at several time intervals to assure equilibrium. Our new data follow:

Table 8. Solubility data on fatty acids in various solvents

(All values in grams acid per 100 gm solution)
(KOLB, 1953)

Temp.	Solvent					
	Methanol	*Ethyl acetate*	*Diethyl ether*	*Acetone*	*Toluene*	*Normal heptane*
			Stearic acid			
10°	0·26	0·58	2·40	0·54	0·0	0·080
0°	0·090	0·13	0·95	0·11	0·080	0·018
− 10°	0·031	0·027	0·38	0·023	0·015	0·004
− 20°	0·011	0·006	0·15	0·005	0·003	—
− 30°	—	—	0·051	—	—	—
			Oleic acid			
− 20°	4·02	5·95	—	5·20	—	2·25
− 30°	0·86	1·90	—	1·68	3·12	0·66
− 40°	0·29	0·62	5·15	0·53	0·96	0·19
− 50°	0·10	0·20	1·80	0·17	0·28	0·050
− 60°	0·03	0·057	0·61	0·055	0·075	0·011
− 70°	—	—	0·21	—	—	—
			Elaidic acid			
0°	—	—	—	—	—	0·59
− 10°	0·48	—	—	—	0·86	0·19
− 20°	0·18	0·29	1·40	0·26	0·20	0·060
− 30°	0·064	0·10	0·60	0·092	0·056	0·019
− 40°	0·020	0·027	0·23	0·029	0·013	0·007
− 50°	0·010	0·008	0·10	0·009	—	—
			Linoleic acid			
− 50°	3·10	4·40	—	4·10	—	0·98
− 60°	0·90	1·38	—	1·20	—	0·20
− 70°	0·25	0·39	—	0·35	—	0·042
			Arachidic acid			
10°	0·080	0·14	0·90	0·13	0·12	0·028
0°	0·028	0·036	0·38	0·035	0·026	0·005
			Eicosenoic acid			
− 20°	0·80	1·30	—	1·10	—	—
− 30°	0·35	0·60	3·90	0·54	1·10	0·45
− 40°	0·15	0·26	1·70	0·27	0·30	0·15
− 50°	0·06	0·11	0·68	0·12	0·07	0·048
− 60°	0·02	0·04	0·22	0·05	—	0·01

Table 8 (continued)

(All values in grams acid per 100 gm solution)

Temp.	Solvent					
	Methanol	*Ethyl acetate*	*Diethyl ether*	*Acetone*	*Toluene*	*Normal heptane*
			Petroselinic acid			
− 10°	—	—	—	—	—	0·50
− 20°	0·45	0·73	3·52	0·78	0·98	0·13
− 30°	0·18	0·30	1·68	0·31	0·23	0·040
− 40°	0·060	0·11	0·76	0·11	0·046	0·009
− 50°	0·018	0·040	0·31	0·035	0·008	—
− 60°	—	—	—	—	—	—
			Petroselaidic acid			
0°	0·50	—	—	—	1·40	0·22
− 10°	0·20	0·36	—	0·32	0·19	0·030
− 20°	0·082	0·14	—	0·13	0·060	0·008
− 30°	0·028	0·050	0·51	0·050	0·020	0·002
− 40°	0·010	0·018	0·19	0·019	0·008	—
− 50°	—	—	0·070	—	—	—
			Stearolic acid			
0°	—	—	—	—	—	—
− 10°	0·65	1·15	4·85	1·26	1·70	0·050
− 20°	0·25	0·45	2·00	0·52	0·40	0·010
− 30°	0·103	0·17	0·78	0·19	0·083	0·006
− 40°	0·040	0·065	0·32	0·070	0·018	—
			Palmitic acid			
10°	1·30	—	—	1·60	1·41	0·30
0°	0·46	0·52	2·95	0·65	0·36	0·08
− 10°	0·16	0·18	1·35	0·27	0·086	0·02
− 20°	0·050	0·060	0·56	0·10	0·018	0·005
− 30°	—	0·018	0·21	0·038	—	—
			Behenic acid			
10°	0·019	0·055	0·48	0·050	0·040	0·012
0°	0·007	0·016	0·18	0·014	0·010	0·002
− 10°	0·002	0·004	0·068	0·004	0·002	—
			Erucic acid			
− 10°	0·49	—	—	—	—	0·35
− 20°	0·19	0·31	—	0·28	0·68	0·11
− 30°	0·068	0·11	1·20	0·10	0·16	0·030
− 40°	0·024	0·040	0·49	0·037	0·044	0·008
− 50°	0·007	—	0·18	—	—	—

Table 8 (continued)

(Ali values in grams acid per 100 gm solution)

Temp.	Solvent					
	Methanol	*Ethyl acetate*	*Diethyl ether*	*Acetone*	*Toluene*	*Normal heptane*
			Brassidic acid			
10°	0·27	0·70	—	0·68	0·62	0·20
0°	0·094	0·26	—	0·24	0·18	0·058
− 10°	0·035	0·096	0·78	0·065	0·050	0·016
− 20°	0·010	0·028	0·28	0·024	0·013	0·005
− 30°	0·003	0·01	0·10	—	—	—

Table 9. Fatty acid solubilities in various hydrocarbon solvents

(All values expressed in grams acid per 100 gm solution)
(KOLB, 1953)

Stearic acid

Temperature	*n-Heptane*	*Methyl cyclohexane*	*n-Pentane*	*Isopentane*
10°	0·080	0·20	0·089	0·096
0°	0·018	0·06	0·015	0·040
− 10°	0·004	0·021	0·003	0·017
− 20°	—	0·005	—	—

Temperature	*Diisopropyl*	*Neohexane*	*2-Methyl pentane*	*Iso-octane*
10°	0·070	0·04	0·086	0·051
0°	0·015	0·010	0·03	0·020
− 10°	0·004	0·003	0·01	0·008
− 20°	—	—	0·003	0·003

Oleic acid

Temperature	*n-Heptane*	*Methyl cyclohexane*	*Diisopropyl*	*Iso-octane*	*Neo-hexane*	*2-Methyl pentane*
− 40°	0·19	0·34	0·212	0·162	0·13	0·19
− 50°	0·050	0·11	0·112	0·070	0·050	0·08

Linoleic acid

Temperature	*n-Heptane*	*Methyl cyclohexane*	*Diisopropyl*
− 50°	0·98	2·06	0·94
− 60°	0·20	0·38	0·17
− 70°	0·042	0·072	0·032

III. Factors Limiting the Application of Solubility Data to the Practical Separation of Mixtures

If the solubility of any given fatty acid in a mixture even approximated its solubility in the pure state, amazing separations would be possible by crystallization procedures. However, association in solution, or intersolubilization, was demonstrated as early as 1919 by Waentig and Pescheck (1919) for a series of saturated acids in various solvents. Inte.solubilization to an important degree was observed in such solvents as chloroform, benzene and nitrobenzene but was insignificant in ethanol, ethyl ether and ethyl acetate. This finding has been extended to mixtures of a saturated and an unsaturated acid by Singleton (1948a and 1948b). A special apparatus (Singleton *et al.*, 1946) was devised for the study of systems containing oleic and stearic and oleic and palmitic acids in acetone and commercial hexane at temperatures from 0° to — 40°. The apparatus permitted equilibration of each system at a given temperature, and separation of crystal and filtrate fractions. Calculation of the compositions of each fraction included corrections to account for entrained filtrate and filtrate acids in the crystal fractions. Even at temperatures which did not permit precipitation of oleic acid (0° and — 10°), small amounts of oleic acid were found to crystallize along with stearic acid (but not with palmitic acid), apparently as mixed crystals. Presence of oleic acid in the filtrates appreciably increased the solubility of the saturated acid. Intersolubilizing effects were greater in hexane than in acetone, although actually the acids are more soluble in acetone than in the former solvent. On the other hand, the presence of stearic acid in solution had little effect on the solubility of the oleic acid. The observed intersolubilizing effect of oleic acid did not support the previous finding by Singleton, Lambou, and Bailey (1945) that the solubility of the saturated fatty acids of cottonseed oil in acetone was little affected by the presence of oleic and linoleic acids. In hexane, on the other hand, the opposite effect was shown.

Quite recently, Schlenker (1952) working with ternary mixtures (oleic, palmitic, and stearic acids) has concluded that the quantities of solid fatty acids dissolved are proportional to the amount of oleic acid present in the mixture.

In addition to intersolubilization and mixed crystal formation, both important effects as evidenced by Singleton's work, there is another important factor which tends to decrease the efficiency of crystallization separations, namely, the fact that crystal fractions from fatty acid mixtures tend to be voluminous, so that complete separation of crystals from mother liquor is difficult. This problem can be overcome in part by redissolving the crystals and recrystallizing one or more times. A simpler scheme is to wash the crystal fraction with solvent which has been previously cooled to a temperature considerably lower than that at which the crystallization has taken place. This conveniently washes out mother liquor from the crystals, but, of course, does not remove any filtrate acids which may have come down as mixed crystals.

IV. Description of a Convenient Laboratory Apparatus for Low Temperature Crystallizations

An apparatus for crystallization at low temperature, which has been in use in this laboratory for many years, is shown in Fig. 1. It consists essentially of a wooden box in which is placed a cylindrical copper can, insulated with rock wool and about half filled with solvent, such as methanol, ethanol, or acetone. The crystallizing vessel, a "Pyrex" cylinder, 150 mm in diameter and 300 mm tall, and weighted with four lead bars, is placed in the solvent. The lead bars serve to prevent the cylinder from capsizing during the later stages of filtration. The solution of fatty acids, up to 4 litres, is introduced into the cylinder. Slow stirring is accomplished by means of an ice cream-type monel paddle. Dry ice is added slowly to the outer solvent and the temperature gradually lowered to the desired level. Temperatures are followed in the bath and in the solution by Weston bimetallic thermometers or other suitable low-temperature thermometers. Inverted suction filtration is carried out through use of a special porcelain funnel* connected to a 4-litre suction flask with neoprene tubing. After placing a circle of filter paper moistened with solvent on the funnel, suction is begun (which holds the paper in place), and the funnel is introduced into the solution from the top. Filtration is expedited by allowing the crystals to settle as much as possible before filtration is begun. Filtration is usually rapid; toward the end of the filtration the crystal layer is pressed in order to permit removal of maximum quantities of mother liquor. It may be desirable to wash the crystals with fresh solvent cooled 10 to 15° below the crystallization temperature.†

V. The Partial Separation of Fatty Acid (or Ester) Mixtures by Low Temperature Crystallization

(a) *Separation of saturated and unsaturated acids*

Brown and Stoner (1937) first used low-temperature crystallization to separate the saturated and unsaturated acids of cottonseed oils. At — 20°, with acetone or methanol as a solvent and a solvent-solute ratio of 10 : 1, they obtained saturated crystal fractions of iodine values 3·4 to 7·2; the separation was claimed to be as efficient as the lead soap-alcohol technique. Earle and Milner (1940) later applied the method to the quantitative estimation of the saturated acids in soybean oil. Their method involved crystallization of 5 gm specimens of the mixed acids three times from 50 ml acetone at — 40°. Since that time, except for a procedure described by DeGray and DeMoise (1941), no detailed general quantitative procedure for separating saturated and unsaturated acids by this technique has been published. On the other hand,

* This funnel (137 mm in diameter) was especially constructed for us by the Coors Co., Golden, Colo. Its stem is tapered out to smaller diameter than standard Buchner funnels, permitting use of smaller bore neoprene tubing. The holes on the funnel are 5 mm in diameter, affording a much larger filtration surface. Filtrations of smaller amounts of solution can easily be performed similarly with sintered glass funnels in cylinders of smaller diameter.

† We also have a deeper crystallizing bath, designed for use of 150 × 450 mm "Pyrex" cylinders with a capacity of 7 litres.

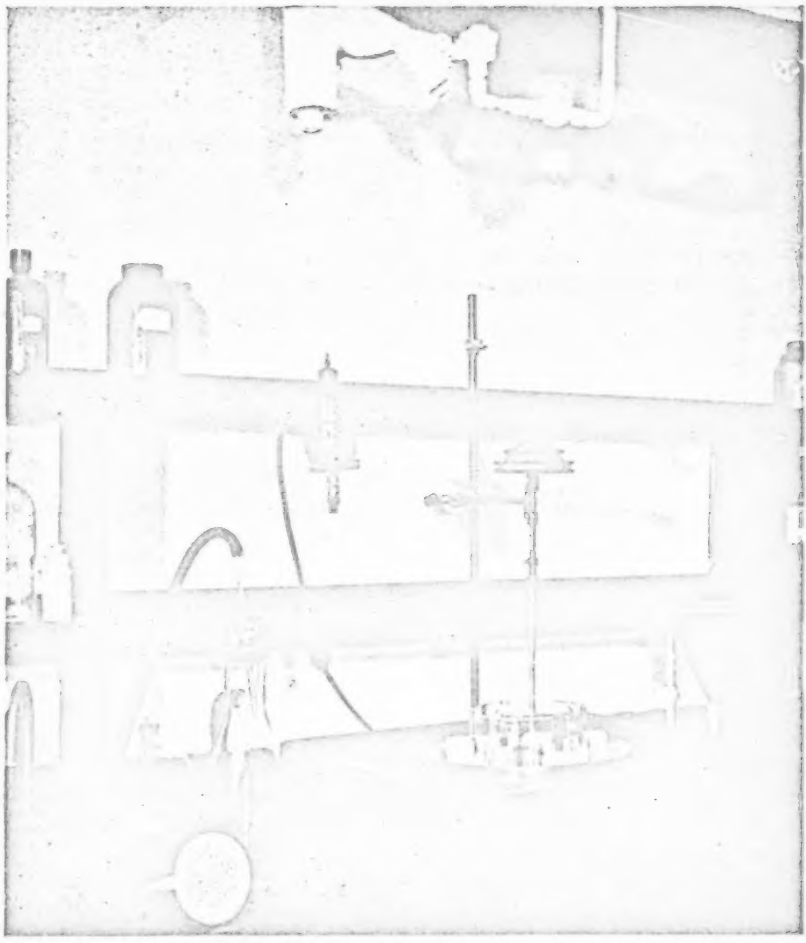


Fig. 1. Apparatus for low temperature crystallization.

various modifications of the method have been used repeatedly to meet specific problems so that it is now the custom, when it is desired to separate the saturated and unsaturated acids of a given fat or oil preliminary to ester distillation, to apply the crystallization method. THORNTON *et al.* (1944) employed a modification of the EARLE and MILNER method to separate the saturated and unsaturated acids of soybean lecithin. SINGLETON and co-workers (1945) separated the fatty acids of cottonseed oil and hydrogenated cottonseed oil by crystallization. Acetone and Skellysolve B were found to be equally effective solvents, but the latter required a temperature about 10°F lower to give an equivalent separation. BALIGA and HILDITCH (1948) twice crystallized the mixed fatty acids of rapeseed oil from ether at — 40°, the crystal fraction containing the saturated acids and most of the erucic acid. The saturated and unsaturated fractions of Buffalo gourd oil were obtained by SHABANI *et al.* (1951) by crystallizing at — 40° from acetone. HANSEN (1951) separated the steam non-volatile acids of butterfat by three crystallizations from acetone at — 30°. The fatty acids of buckwheat leaf meal fat were similarly separated by KREWSON (1952).

The preceding examples suffice to illustrate applicability of the method to the usual types of fat, to a fat rich in erucic acid and to a fat containing a wide range of carbon series, such as butterfat. In applying the method, one should keep in mind the nature of the fatty acid mixture being investigated and the solubilities of the fatty acids in the mixture. By this procedure lauric acid, and to an appreciable extent myristic acid, may be found in the unsaturated fraction. Hydrogenated fats and fats rich in erucic acid give fatty acid mixtures of intermediate solubility, so that special procedures have to be devised to achieve workable separations. In a fatty acid mixture consisting primarily of myristic, palmitic, stearic, hexadecenoic, oleic, linoleic, and linolenic acids, simple crystallization of 5 per cent solutions of the mixed acids from acetone at — 25°, followed by a single washing of the crystals with solvent cooled to about — 40°, may be expected to provide a more simple and more efficient separation than can be accomplished by the lead salt method.

An important commercial method of selective solvent separation of fatty acids is the so-called "Emersol Process," for the following description of which we are indebted to Dr. V. K. MUCKERHEIDE of Emery Industries.

The Emersol Process (MYERS and MUCKERHEIDE, Patents, 1942 and 1947; KISTLER *et al.*, 1946) involves the controlled crystallization of fatty acids from a polar solvent to achieve a separation of the fatty acids. Although more particularly adapted to the separation of solid fatty acids (saturated) from liquid fatty acids (unsaturated) the Emersol Process can also be used for the separation of saturated acids or the separation of triglycerides (MUCKERHEIDE, Patent, 1950). A typical illustration of the application of the process is in the separation of animal fatty acids to yield commercial stearic acid and oleic acid. Animal fatty acids properly prepared are dissolved in 90 per cent methanol to give a 25 to 30 per cent concentration. This solution is then continuously chilled in a multi-tubular, agitated crystallizer to about — 15°. In this chilling

the solid acids crystallize from the solution to form a slurry which in turn is fed to a continuous, rotary vacuum filter. The solid acids are formed into a cake, which is continuously washed with methanol and subsequently discharged from the filter. This filter cake, containing about 50 to 60 per cent of solvent, is melted and pumped to a solvent recovery still (BROWN, Patent, 1943) in which the solvent is removed and returned to the system. The solid acids, stearic acid, are discharged from the still ready for finishing and packaging operations. The filtrate, containing the liquid acids, is passed through a heat exchanger to another solvent recovery still from which the liquid acids, "oleic acid," are discharged. Typical rates of through-put range from thirty (30) to fifty (50) tons per day, depending on the size of the Emersol Unit and the character of the fatty acids being processed.

Typical results achieved in the separation of various types of fatty acids are tabulated:

Type of fatty acid	*Titre*	*Iodine value*	*Products*	*Yield*	*Titre*	*Iodine value*
Animal (tallow)	42·4°C	52·2	Stearic	51%	54·4°	6·0
			Oleic	49	2·0°	
Cottonseed	—	103·0	Solid acids	30	—	7 to 12
			Liquid acids	70	—	142 to 144

The first commercial unit of the Emersol Process has been in operation since 1942. At the present time there are a total of five (5) units in operation in the United States, Great Britain, and Australia.

Other procedures for separating saturated and unsaturated acids have been patented by TOWNE (1949) and FELDPUSH (1950).

(*b*) *The separation of unsaturated acid mixtures*

Low temperature crystallization is admirably adapted to the further separation of unsaturated fatty acid mixtures. Thus, it has been used repeatedly in the evaluation of the component acids of fats to provide fatty acid fractions of simpler composition than the original mixture of acids. Examples illustrating this use of the method were given in the previous review (BROWN, 1941). However, impetus to this use came from a series of papers from HILDITCH's laboratory, beginning in 1945. For fats in which oleic and linoleic acids are major components, HILDITCH and RILEY (1945) proposed the following separation scheme: "The mixed acids are first crystallized from acetone (5 cc per gm) at — 30° when the portion left in solution is found to contain most of the linoleic acid. The deposited solids are then crystallized from ether (10 cc per gm) at — 30° when the soluble material consists of oleic and linoleic acids with minor proportions of saturated acids; the deposited solids should have a comparatively low iodine value (10 to 20), due as a rule wholly to oleic acid." Several variations of this procedure were applied to sunflower, sesame, and peanut oils;

each time three or four sub-fractions were first obtained, which were subsequently converted to methyl esters and fractionally distilled. GUNSTONE and HILDITCH (1946) applied the method to oils containing linolenic acid (cf. SHINOWARA and BROWN, 1938); by crystallizing the fatty acids of linseed oil at — 50° from acetone and ether they obtained four fractions of iodine values 21·2 to 235·2. The method was modified by HILDITCH and RILEY (1946) for use on fats containing eleostearic acid and by HILDITCH and PATHAK (1947) for the study of the acids of marine animal oils. GUNSTONE and PATON have similarly studied the acids of deer and camel fats (1953a) and of python fat (1953b). By crystallization of the mixed acids of seal oil from acetone at — 60° and — 40° and from ether at — 30° four fractions of iodine values 2·8, 75·6, 103·1, and 294·4 were obtained. Many other investigations by HILDITCH and co-workers and by other investigators in the field have regularly employed the procedure or some modification thereof. (Cf. GUNSTONE and PATON, 1953a.)

The separation of the fatty acids of menhaden oil by fractional crystallization of a solution of 100 gm of the acids in Skellysolve B was described by BROWN (1944). Crystal fractions were removed at — 10°, — 20°, — 30°, — 40°, and — 65°. The composition of each of the crystal fractions was calculated, corrections being applied for entrained mother liquor acids. Iodine values of the three intermediate crystal fractions ranged from 4·6 to 9·0, suggestive of deposition of small amounts of monoethenoic acids as mixed crystals in each of these fractions. The crystal fraction at — 65° gave an iodine value of 86·3, very close to that of oleic acid. The — 65° filtrate, iodine value 269, consisted of a mixture mainly of polyethenoic acids capable of esterification with glycerol to make an excellent drying oil.

It might be pointed out that crystallization procedures have not yet been successfully applied to mixtures of unsaturated fatty acids with four or more double bonds. Since these acids are very low melting (arachidonic, — 49°), it is understandable that their solubilities are extremely high even at — 75°, the practical working limit with dry ice. Crystallization methods for these acids will probably necessitate the use of a coolant such as liquid air.

(c) *The separation of fatty acid mixtures by crystallization of methyl esters*

In attempting to separate fatty acid mixtures, it is often preferable to work with methyl esters rather than with the free acids, because association in solution is practically non-existent with esters. On the other hand, esters present the disadvantage of being more soluble than the acids, necessitating the use of lower temperatures. We have observed, for example, that, while it is easy to crystallize linolenic acid from solvent at — 75°, the methyl and ethyl esters of this acid are still very soluble and hence do not crystallize easily at this temperature. When it is desired, therefore, to work with esters of this type, one must consider the use of temperatures perhaps of the order of — 100°. Few solvents are suitable for work at this temperature.

BROWN and STONER (1937) first described the partial separation of the

unsaturated methyl esters of cottonseed oil to give filtrate fractions containing 82 per cent methyl linoleate. De la Mare and Shorland (1944) obtained liquid and solid acid fractions of pig back fat by crystallizing the mixed methyl esters three times from acetone (15 ml per gm) at — 35°. Abu-Nasr and Potts (1953) applied this procedure to the esters of the oil of Citrullus colocynthis. In comparing their results with those obtained by the lead soap method they concluded that "the technique of low temperature crystallization is advantageous not only by reason of its simplicity and directness but also due to its efficiency as evidenced by an iodine value of 7·2 for the saturated fraction."

The separation of the methyl esters of menhaden oil into saturated, monoethenoic and polyethenoic fractions by crystallization was reported by Smith and Brown (1945a). By cooling 100 gm of the mixed esters in 4 l. acetone to — 55° and recrystallizing the crystal fraction twice from methanol, a saturated fraction of iodine value 5·9 and a combined filtrate fraction of iodine value 234·1 were obtained. The filtrate fraction in methanol (10 per cent solution) at — 70° yielded a further crystal fraction containing additional saturated esters and most of the monoethenoic esters. These were repeatedly crystallized from methanol. As a result of a series of crystallizations 1700 gm of menhaden oil esters was separated into the three fractions described in Table 10. Distillation of the monoethenoic fraction described above showed it to be contaminated

Table 10. Separation of menhaden oil esters into saturated, monoethenoic, and polyethenoic fractions

(Smith and Brown, 1945a)
Menhaden oil esters, 1700 gm

Saturated	*Monoethenoic*	*Polyethenoic*
489·0 gm (30·9%) I.N. 4·8 P.N.* 0·0	*239·0 gm* (15·1%) I.N. about 95 P.N.* 3·9	*853 gm* (54·0%) I.N. 285·2 P.N.* 80·5

* Polybromide No.

with small amounts of methyl myristate, which, on account of its solubility and possible intersolubilization, remained in the filtrates even at the low temperatures used. It was further observed that recovery of the monoethenoic esters was incomplete due to intersolubilization with the polyethenoic esters in the several filtrates. By this technique it was possible to show the presence in this oil of monoethenoic acids of series C_{14} to C_{22}. Winter and Nunn (1950) obtained ester fractions of elephant seal oil by crystallization from acetone at — 20°, — 50°, and — 80°; iodine values, 19·0 to 231·0.

One of the most important applications of crystallization of methyl esters is that of separation of the components of single carbon series following fractional ester distillation. We have selected a number of examples illustrative of this point from work done in this laboratory. Numerous other examples could be cited.

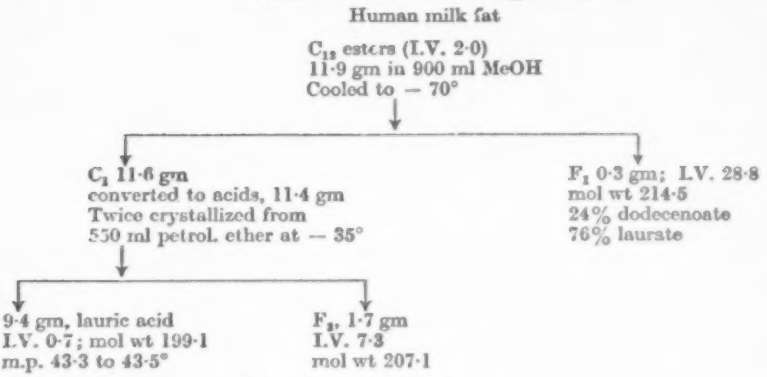
Separation of C_{12} Esters of Human Milk Fat. (BROWN and ORIANS, 1946)

A small C_{12} fraction, 11·9 gm of I.V. 2·0, was separated as shown in Chart 1. The dodecenoate in F_1 had been concentrated about twelve times; in F_2, about three times.

CHART 1

CRYSTALLIZATION OF C_{12} ESTERS

Human milk fat



Separation of Methyl Myristate and Tetradecenoate

The C_{14} esters of human body fat contain about 6 per cent of methyl tetradecenoate. A similar mixture from butterfat was separated by BOSWORTH and BROWN (1933) by bromination and fractional distillation to obtain methyl myristate and dibromomyristate which was subsequently reduced to tetradecenoate. This separation was more conveniently accomplished by CRAMER and BROWN (1943) as follows (Chart 2):

CHART 2

CRYSTALLIZATION OF C_{14} ESTERS

Human Depot Fat

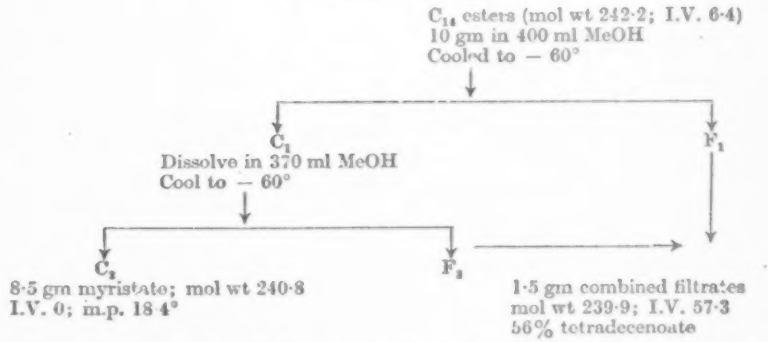


Chart 2 describes a ten-fold concentration of methyl tetradecenoate and the isolation of nearly pure methyl myristate in three operations. An equally efficient separation is described by BROWN and ORIANS (1946) working with human milk fat.

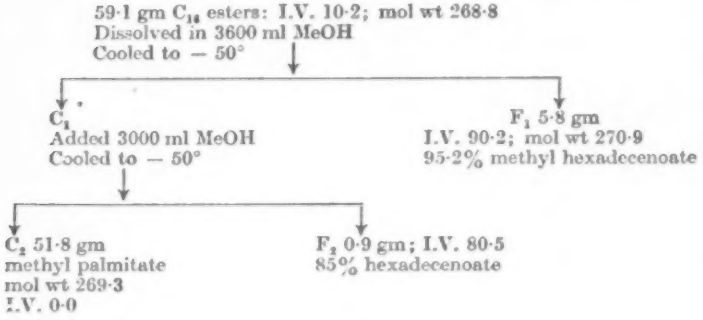
Separation of Methyl Palmitate and Hexadecenoate

The C_{16} ester fraction of human milk fat (BROWN and ORIANS, 1946), containing about 10 per cent of methyl hexadecenoate, was separated as shown in Chart 3. Two crystallizations gave nearly pure methyl palmitate and a fraction containing methyl hexadecenoate of 95·2 per cent purity.

CHART 3

CRYSTALLIZATION OF C_{16} METHYL ESTERS

Human milk fat

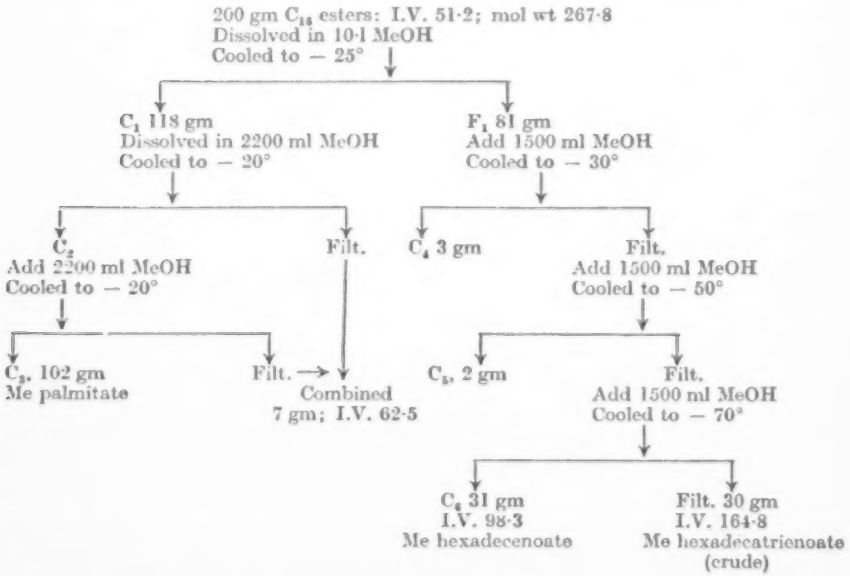


Separation of Methyl Palmitate, Hexadecenoate, and Hexadecatrienoate

A specific problem arises in the separation of the C_{16} ester fraction of marine animal oils, which contains not only palmitate and hexadecenoate, but also

CHART 4

CRYSTALLIZATION OF C_{16} METHYL ESTERS MENHADEN OIL



hexadecatrienoate. A procedure which resulted in the isolation of pure methyl palmitate, and crude specimens of other C_{16} monoethenoic and trienoic esters from menhaden oil is described in Chart 4 (SMITH and BROWN, 1945b).

Separation of C_{18} Esters

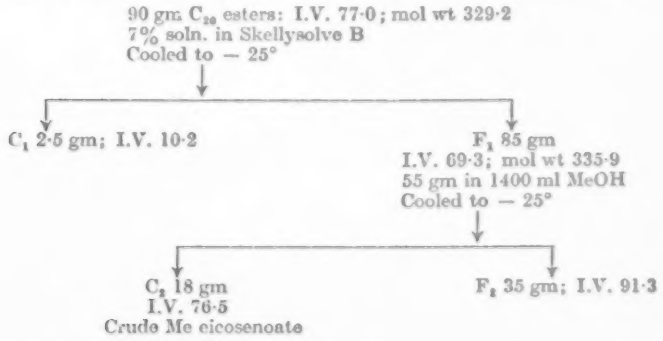
Details of crystallizations of the C_{18} esters of human fat, human milk fat, and menhaden oil are given in the references cited above. The scheme employed by FOREMAN and BROWN (1944) for the isolation of pure methyl oleate is described in a later section of this review. In a simple mixture of stearate, oleate, and linoleate, it is very easy by several crystallizations from methanol at — 60° to remove all of the linoleate. Most of the stearate can then be taken out by allowing a 2·5 per cent solution of the esters in methanol to stand at — 25°. The efficiency of this latter separation is illustrated by the following example (FOREMAN, 1942): '100 gm of a mixture of stearate and oleate of iodine value 82 (about 96 per cent oleate) was allowed to stand in 4 l methanol for 1½ days at — 25°. Large flat crystals of methyl stearate (3·5 gm; I.V. 4·0) came down under this treatment'. If, on the other hand, the fatty acid mixture contains also linolenate, it is easy to remove the stearate and most of the oleate, but it is not possible by crystallization to fully separate the linoleate and linolenate, and thus to purify methyl linolenate or the free acid by this method. For additional applications of separation of C_{18} methyl ester fractions, see the paper by MILLICAN and BROWN (1944), who applied the method to the C_{18} fractions of a large series of animal and vegetable fats.

Separation of C_{20} Esters

FOREMAN (1942) separated the C_{20} esters of rapeseed oil into methyl arachidate, eicosenoate, and possibly eicosadienoate by the following scheme (Chart 5):

CHART 5

SEPARATION OF C_{20} METHYL ESTERS OF RAPESEED OIL

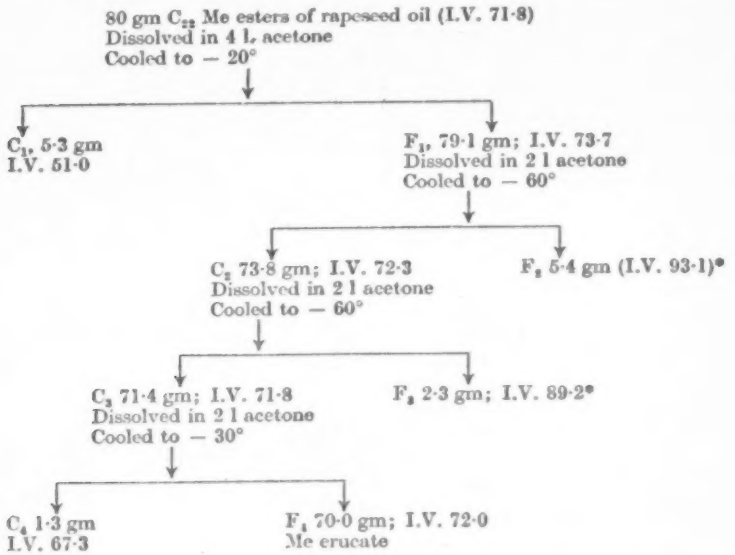

90 gm C20 esters: I.V. 77·0; mol wt 329·2
7% soln. in Skellysolve B
Cooled to — 25°
C1 2·5 gm; I.V. 10·2
F1 85 gm
I.V. 69·3; mol wt 335·9
55 gm in 1400 ml MeOH
Cooled to — 25°
C2 18 gm
I.V. 76·5
Crude Me eicosenoate
F2 35 gm; I.V. 91·3

Separation of C_{22} Esters

By use of a modification of a method previously employed by FOREMAN (1942), KOLB (1953) separated the C_{22} methyl esters of rapeseed oil as described in Chart 6.

CHART 6

SEPARATION OF THE C_{22} ESTERS OF RAPESEED OIL


80 gm C22 Me esters of rapeseed oil (I.V. 71·8)
Dissolved in 4 l. acetone
Cooled to − 20°
C1, 5·3 gm
I.V. 51·0
F1, 79·1 gm; I.V. 73·7
Dissolved in 2 l acetone
Cooled to − 60°
C2 73·8 gm; I.V. 72·3
Dissolved in 2 l acetone
Cooled to − 60°
F2 5·4 gm (I.V. 93·1)*
C3 71·4 gm; I.V. 71·8
Dissolved in 2 l acetone
Cooled to − 30°
F3 2·3 gm; I.V. 89·2*
C4 1·3 gm
I.V. 67·3
F4 70·0 gm; I.V. 72·0
Me erucate

In the examples cited above the value of low temperature crystallization as a method of separating methyl esters of specific carbon series from C_{12} to C_{22} is amply demonstrated. Generally, better separations are possible with esters than with acids for separating saturated from monoethenoic acids and monoethenoic from diethenoic acids. On the other hand, difficulty is usually experienced in separating mixtures of di-, tri-, and polyethenoic esters with four and five double bonds. With these latter compounds intersolubilization and eutectic formation are potential barriers to efficient separations.

VI. The Isolation of Pure Fatty Acids and of Concentrates of Certain Polyethenoic Acids

Crystallization at low temperatures is admirably adapted to the isolation of pure fatty acids and many types of fatty acids have been prepared by various modifications of the procedure. Actually, several of the charts appearing in the previous section illustrate procedures which have resulted in relatively pure acids or esters. Some of the principles involved in the isolation of different types of naturally occurring acids are discussed below with specific illustrations.

(*a*) *Saturated acids and esters*

Homologous series of acids or esters differing by only two carbon atoms are extremely difficult to separate by simple crystallization, their solubilities being too nearly alike. On the other hand, if one component of a binary mixture of two adjacent saturated acids is present in large excess and the major

* F_2 and F_3 contain appreciable amounts of polyethenoic esters.

component is the less soluble, the separation can be accomplished simply by a series of crystallizations. Even if the major component is the more soluble, part of it can eventually be obtained pure, provided the less soluble component is present in small enough amount. Thus, if the solubility ratio is 5 : 1 and only 5 per cent of the less soluble component is present, the more soluble component can be crystallized under such conditions as to leave the unwanted component in solution. In attempting such a separation, esters should be employed instead of the free acids to minimize mixed crystal formation.

As a rule, the first step in preparing a pure saturated acid such as stearic acid or its methyl ester is to isolate the pure C_{18} methyl ester fraction from a fat rich in stearic acid by ester distillation. All unsaturated esters can then be easily removed by several crystallizations from petroleum ether, acetone, or methanol at $-20°$. The more dilute the solution the more efficient will be each crystallization. For acids of lower molecular weight, such as capric or lauric, the corresponding C_{10} or C_{12} fractions of butterfat or coconut oil are employed, but it must be kept in mind that these esters are decidedly more soluble at $-20°$ than C_{16} or C_{18} esters and temperatures down to $-40°$ or lower will be necessary.

(*b*) *Oleic and other monoethenoic acids*

The simplest method of preparing oleic acid of 95 to 98 per cent purity is the method of Brown and Shinowara (1937), who started with the mixed fatty acids of olive oil. Saturated acids were largely removed by cooling an acetone solution of these acids to $-20°$. Upon further cooling the filtrate to $-65°$ the crystal fraction contained most of the oleic acid. Three additional crystallizations at this temperature resulted in complete removal of linoleic acid. The final product in this instance contained small amounts of palmitic acid. The Foreman and Brown (1944) procedure, cited previously, is very similar to that used by Wheeler and Riemenschneider (1939), and is directed first to the isolation of pure methyl oleate, from which oleic acid can be obtained by the usual methods. This method involves (1) preparation of pure C_{18} methyl esters of olive oil by twice distilling through a packed column; (2) five crystallizations from methanol to remove all linoleate; (3) crystallizing at about $-30°$ from methanol to remove remaining amounts of stearate. Many subsequent workers have employed essentially this same method (Stirton *et al.*, 1945; Swern *et al.*, 1944; Lutton, 1946; Paquot and Durrenberger, 1951; Feuge *et al.*, 1951; Ross *et al.*, 1949; Golumbic, 1946; Swern *et al.*, 1946; Swern *et al.*, 1945; Holman and Elmer, 1947). It should be cautioned that oleic acid prepared from animal fats is likely to contain small amounts of isooleic acids (Swern *et al.*, 1946), including variable amounts of *trans* acids. The latter may occur in large amounts in shortenings and margarines made from hydrogenated fats (Mabrouk, 1953).

Variations of the Foreman-Brown and Wheeler and Riemenschneider techniques are suitable for the isolation of practically any of the monoethenoic acids, provided the appropriate carbon series is first isolated by ester

distillation. Thus, HOPKINS *et al.* (1949) obtained pure methyl eicosenoate by crystallizing the C_{20} esters of jojoba oil from acetone at — 40° and recrystallizing at — 15°. The preparation of 11-eicosenoic acid has been previously mentioned (FOREMAN and BROWN, 1944). Details of an improved method of preparing pure ricinoleic acid are described by HAWKE (1950).

(c) *Linoleic acid*

In the previous review (BROWN, 1941) the preparation of linoleic acid by repeated crystallization of alpha-linoleic acid was cited. The iso-linoleic acids which are found in alpha-linoleic acid are much more soluble than linoleic acid and repeated crystallization of the mixture from acetone suffices to remove them.

BAUR, in our laboratory (1943), repeatedly crystallized beta-linoleic acid from petroleum ether and succeeded in isolating two components, one of which was identical with alpha-linoleic acid, containing about 90 per cent of linoleic (*cis,cis*-9,12-octadecadienoic) acid, and the other a mixture of iso-acids, which yielded no petroleum ether-insoluble tetrabromostearic acid.

A more simple method of preparing linoleic acid is to directly crystallize the fatty acids of an oil rich in linoleic acid and containing no linolenic acid, as described by FRANKEL and BROWN (1941): this method was applied later to a series of oils by FRANKEL, STONEBURNER, and BROWN (1943). A brief description of the method follows:

'(1) The mixed fatty acids of the oil are dissolved in acetone (75 gm per litre solvent) and cooled to — 20 to — 25°. The crystal fraction is removed by inverted suction filtration.

(2) The filtrate from (1) is further cooled to — 50° with slow stirring and is filtered, as before.

(3) The filtrate from (2) is cooled to — 70°. The crystal fraction is about 90 per cent linoleic acid.

(4) The crystal fraction from (3) is dissolved in 30° to 60° petroleum ether, 65 gm per litre and cooled to — 48°. The crystals at this temperature are usually 95 per cent linoleic acid. At — 48° the solubilities of oleic and linoleic acids are more nearly equal in petroleum ether than in acetone, so that relatively larger amounts of the oleic acid remain in solution.

(5) The 95 per cent acid is dissolved in petroleum ether, 6·25 gm per litre, and cooled to — 60° to — 62°. This time the crystal fraction is practically pure linoleic acid. The objective of this step is to use enough solvent to keep all of the oleic acid in solution, and thus to crystallize pure linoleic from the solution. The success of the last operation depends on the fact that the starting material is a 19 : 1 linoleic/oleic acid mixture, while at — 60° the ratio of solubilities is very much smaller. Very low concentrations also tend to minimize mixed crystal formation'. By this procedure FRANKEL *et al.* successfully isolated linoleic acid from sesame, cottonseed, grapeseed, and poppyseed oils; subsequently, it has been used successfully in our laboratory to prepare pure linoleic acid from the fatty acids of soybean lecithin, and soybean, wheat germ, tobacco seed, and safflower oils. Whenever the original fat contains

significant amounts of iso-linoleic acids, as in butterfat, human milk fat, human fat, and olive oil, the product is usually found to be a mixture of oleic, linoleic, and iso-linoleic acids, probably due to intersolubilization or to eutectic formation. Partial separation of such a mixture can be accomplished by chromatography as shown by LEMON (1949). Other modifications of low temperature crystallization methods as applied to isolating linoleic acid are described by HILDITCH, PATEL, and RILEY (1951) and PAQUOT and MERCIER (1951). A concentrate of docosadienoic acid was obtained by crystallization of the C_{22} esters of rapeseed oil from acetone at — 60° (BALIGA and HILDITCH, 1949). Employing a combination of the ester distillation and low temperature crystallization techniques, CROSSLEY and HILDITCH (1950) partially separated the complex fatty acid mixtures of stillingea oils and obtained concentrates of the unique 2,4-decadienoic acid which occurs in this oil to the extent of about 5 per cent.

(*d*) *Linolenic and other trienoic acids*

Pure linolenic acid has been prepared by repeated crystallization of debromination linolenic acid as described by MATTHEWS, BRODE, and BROWN (1941). However, a recent attempt in our laboratory to repeat this work failed because the debromination acid, although its iodine value was close to the theoretical, contained about 34 per cent of iso-linolenic acids which are about three to four times as soluble as linolenic acid. On each attempt to crystallize at — 75° the composition of the filtrate acids and crystals remained about the same. This result may have been due to eutectic formation. No crystallization procedure, starting with acid mixtures from natural sources, such as linseed or perilla oils, has resulted so far in a product more than 90 to 92 per cent pure. It is very easy by starting with the acids of linseed oil and freezing out lesser unsaturated acids to obtain good yields of 75 per cent linolenic acid; it is possible but much more difficult, for the reasons mentioned previously, to get a product of 90 per cent purity. In the earlier stages of the process it is preferable to use methyl or ethyl esters, but as the product becomes purer, the separation becomes more difficult. Furthermore, solubilities of methyl and ethyl linolenates are so high that temperatures lower than — 75° would seem to be necessary. Preparation of concentrates of linolenic acid have been described by the following: (SHINOWARA and BROWN, 1938; HILDITCH *et al.*, 1951; RUSOFF *et al.*, 1945). KNIGHT *et al.* (1946) studied concentrates of linoleic and linolenic acids, which they isolated from beef tallow acids by an extended series of crystallizations from acetone. A concentrate of hexadecatrienoic acid was prepared by crystallizing the C_{16} methyl esters of rape leaf lipids from methanol at — 15° by SHORLAND (1945). (Cf. also SMITH and BROWN, 1945b.) By crystallization of the acids of the seed fat of the evening primrose RILEY (1949) was able to isolate and identify 6,9,12-octadecatrienoic acid.

(*e*) *Other polyethenoic acids*

Up to the present, as noted previously, low temperature crystallization has not been successfully used in the isolation of pure arachidonic acid or of other

polyethenoic acids with four, five, and six double bonds. Concentrates of methyl arachidonate (65 to 70 per cent) may be easily obtained by cooling solutions of the methyl esters of the fatty acids of adrenal phosphatides in acetone or methanol to — 75° (MOWRY *et al.*, 1942). Recently, methyl arachidonate of about 92 per cent purity was obtained by fractional distillation of such a concentrate (WHITE, 1949). The pure ester has been isolated by chromatography (WHITE and BROWN, 1948; HERB *et al.*, 1951). Purified α- and β-parinaric acids and some of their derivatives were prepared from the oil seeds of Balsaminaceae by crystallization of the acids from petroleum ether (KAUFMANN and KELLER, 1950). SHORLAND and co-workers have repeatedly used low temperature crystallization in the course of their investigations of New Zealand butterfat (HANSEN and SHORLAND, 1950, 1951; SHORLAND, 1950). More particularly, SHORLAND and JOHANNESON (1951) isolated a C_{20-22} methyl ester fraction which they crystallized from acetone at — 50° to yield a filtrate fraction of iodine value 216·5. This, as well as a number of similar preparations, was shown to be a concentrate of both tetraene and pentaene esters of these series.

VII. Separation of Glycerides by Low Temperature Crystallization

As noted in the previous review (BROWN, 1941), the early attempts to separate the glyceride mixtures of the natural fats were unsuccessful because these glycerides do not occur as monoacid triglycerides but rather, as proposed by HILDITCH (1941), as mixed triglycerides, in which the fatty acids are distributed, according to the so-called "even distribution rule" or, as proposed by others, according to a random pattern. NORRIS and MATTILL (1947) have claimed that fats do not adhere strictly to either of these theories of structure, but that animal fats tend to approach random distribution while seed fats more nearly approximate even distribution. DOERSCHUK and DAUBERT (1948) have shown that a partial random scheme best explains the glyceride structure of corn oil. According to any of these theories (except the monoacid), a major component acid of a given fat should be found distributed over almost all of the glyceride molecules present. As early as 1901 HOLDE and STANGE (1901) crystallized an ether solution of olive oil at — 40° and demonstrated for the first time that this oil, in spite of high content of oleic acid, contains no significant amount of triolein.

In the late 1930's, HILDITCH and his associates developed the use of low temperature crystallization as a part of their technique for determining the glyceride composition of various fats. The method was first applied to cottonseed oil (HILDITCH and MADDISON, 1940) and has since been used extensively in this laboratory for studying the glycerides of a large number of fats and oils, including palm oil (HILDITCH *et al.*, 1947a; MEARA, 1948), cacao butter (MEARA, 1949), human milk fat (HILDITCH and MEARA, 1944), and various marine oils (HILDITCH and MADDISON, 1948; HILDITCH and PATHAK, 1947; BJARNASON and MEARA, 1944), animal fats (HILDITCH and SHRIVASTAVA, 1949; GUPTA *et al.*, 1950), seed oils (HILDITCH and MADDISON, 1940, 1941;

GUNSTONE *et al.*, 1947; HILDITCH *et al.*, 1947b; DUNN *et al.*, 1948; HILDITCH *et al.*, 1947c; CROSSLEY and HILDITCH, 1953), and drying oils (HILDITCH and RILEY, 1946; HILDITCH and SEAVELL, 1950; HILDITCH, 1949; BARKER and HILDITCH, 1950; HILDITCH and MENDELOWITZ, 1951). The procedure generally involves separation of the glycerides into about five or six fractions by crystallization from acetone at temperatures lying between 10° and — 60°. A typical fractionation scheme might involve successive separations at — 60°, — 40°, — 20°, and — 10°. It is generally possible to separate glyceride mixtures into the following series of fractions: GS_3, GS_2U, GSU_2, and GU_3, where S and U represent saturated and unsaturated fatty acids, respectively, and G designates the glycerol moiety. A rather interesting application of the method was the crystallization of the perinephric fat of a pig which had been fed on a diet of whale oil (GORTON, HILDITCH, and MEARA, 1952). Crystallization from acetone at — 40° yielded 26 per cent of a soluble fraction which was similar in composition to whale oil and 74 per cent of an insoluble fraction which resembled normal pig fat. The preparation of certain glyceride concentrates from a large series of natural fats and oils is described by CAMA *et al.* (1953).

A detailed fractionation of butterfat was reported (HENDERSON and JACK, 1944; JACK and HENDERSON, 1945; JACK *et al.*, 1946). The fat was crystallized from pentane at — 7°, — 13°, — 23°, and — 53°, yielding four crystal fractions and a filtrate; the corresponding iodine values were 8·3, 22·2, 23·5, 31·2, and 58·4. "Solid" and "liquid" acids were prepared from each fraction by freezing from acetone at — 20°. All ten "liquid" and "solid" fractions were submitted to ester distillation. It is interesting to note that when the five glyceride fractions were fed to weanling rats (JACK *et al.*, 1945) the — 53° filtrate gave the best growth response and the — 7° precipitate the poorest.

RIEMENSCHNEIDER and co-workers (1946) fractionated lard and tallow by crystallization from acetone. The resulting seven fractions from lard, obtained at temperatures from 20° to — 45°, gave iodine values of 4·5 to 130·6. Spectroscopically, the final filtrate fraction was shown to contain 35·75 per cent linoleic acid, 2·40 per cent linolenic acid, and 4·69 per cent arachidonic acid. Cottonseed oil (RIEMENSCHNEIDER *et al.*, 1940) was similarly separated into seven fractions by use of temperatures down to — 65°. Low temperature crystallizations of corn oil into nineteen fractions (DOERSCHUK and DAUBERT, 1948) and of soybean oil (DUTTON *et al.*, 1950) were carried out in connection with studies of glyceride structure of these oils.

CORDING and co-workers studied the conditions for fractionating white grease by solvent crystallization, using both anhydrous (CORDING *et al.*, 1953a) and water-saturated (CORDING *et al.*, 1953b) ketones and esters as solvents. Liquid propane (LACEY *et al.*, 1951) has also been used as a fractionating solvent for glycerides.

A commercial process has been described (JAMES, 1949) for separating soybean oil into two fractions, a more saturated fraction suitable for edible products and soaps and a less saturated fraction for use in such commodities as

paints and varnishes. The oil was mixed with one or two parts solvent and cooled rapidly to − 20° to − 60°F, the precipitated material being removed by continuous centrifugation. Crystallization at low temperatures has also been used successfully by DAUBERT and co-workers (JACKSON *et al.*, 1944; DAUBERT and BALDWIN, 1944; DAUBERT and SIDHU, 1948) for purifying certain synthetic glycerides.

Several investigators have determined solubilities for various purified triglycerides, but the information relates almost entirely to the saturated compounds. LOSKIT (1928) reported the solubility behaviour of the simple saturated triglycerides from tricaprin to tristearin. His work covered rather wide ranges of temperature and included a number of organic solvents. Fewer data are available for mixed triglycerides; however, certain values for both symmetrical and unsymmetrical diacid triglycerides have been reported (ROBINSON *et al.*, 1932; MCELROY and KING, 1934), while CHEN and DAUBERT (1945) have determined solubilities for a series of triacid triglycerides. The solubilities of certain glycerides in liquid propane were investigated by HIXON and co-workers (HIXON and HIXON, 1941; HIXON and BOCKELMAN, 1942; DREW and HIXON, 1944). Their study included tristearin, tripalmitin, tricaprylin, and refined cottonseed oil in various binary and ternary systems. Although solubilities for the fully unsaturated glycerides have not yet been determined, their relative values can be roughly estimated from a consideration of their melting points.

VIII. MISCELLANEOUS APPLICATIONS

In the section which follows are described miscellaneous applications of low temperature crystallization to the separation of a wide variety of mixtures.

(*a*) *The preparation of pure egg lecithin*

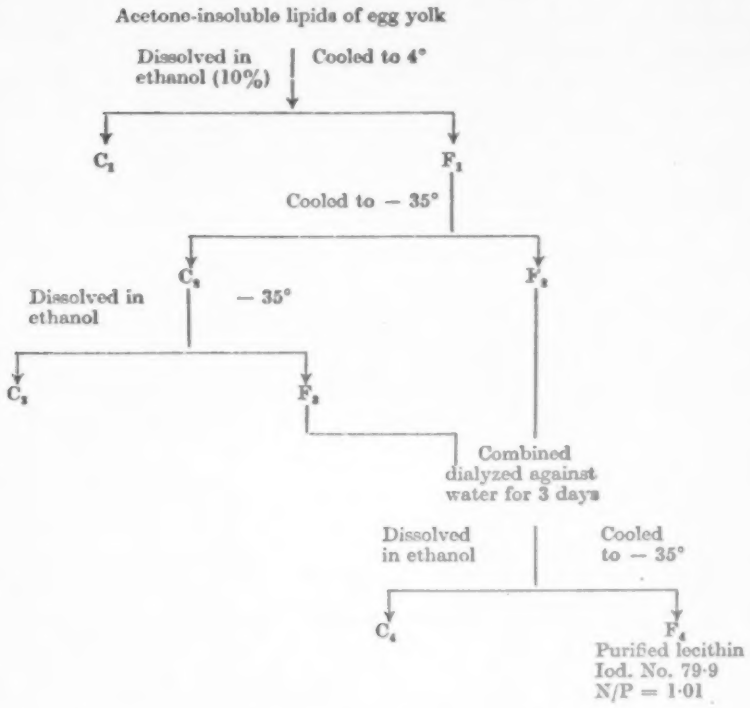
It is strange that low temperature crystallization has as yet been used only infrequently for the separation of other lipid mixtures than those described previously. However, one such application, Chart 7, is the preparation of pure lecithin from egg lipids by SINCLAIR (1948). The product had a considerably higher iodine value than one prepared by conventional methods.

(*b*) *Synthetic fatty acids and fatty acid derivatives*

Numerous examples of the successful application of low temperature crystallization to the separation of synthetic fatty acids and certain derivatives of the fatty acids have appeared in the literature. While space does not permit mention of all of these references, some of the more important typical uses of the procedure follow.

CASON and co-workers have isolated certain branched chain acids. For example, 15-methyl octadecenoic acid (CASON and COAD, 1950) was purified by crystallization from acetone at − 25°; successive crystallizations at − 30° and − 70° were likewise used to purify 9-*n*-octyl heptadecanoic acid (CASON and STANLEY, 1949).

CHART 7
PREPARATION OF EGG LECITHIN



The method has proved convenient for separating *trans*-fatty acids from their equilibrium isomerization mixtures. Elaidic acid, for example, is readily crystallizable from an oleic-elaidic mixture by cooling a 10 per cent acetone solution of the mixed acids to about − 20°. Crystallization at − 12° was reported effective for separating petroselaidic acid from the products obtained by the action of N_2O_4 on petroselinic acid (WILLIAMS and VASIL'EV, 1948).

FUSARI *et al.* (1951) applied low temperature crystallization to the purification of a series of synthetic "iso-oleic" acids, including *cis*-7-, *trans*-7-, *cis*-8-, and *trans*-8-octadecenoic acids, as well as both the *cis*- and *trans*-11-octadecenoic (vaccenic) acids. They also utilized the method in purifying certain intermediate compounds, such as the 7- and 8-octadecynoic acids. Additional *trans* acids which have been prepared in this laboratory using the low temperature crystallization technique include brassidic, *trans*-eicosenoic, and *trans*-hexadecenoic acids. NICHOLS *et al.* (1951) have published a detailed scheme for separating the isolinoleic acids found in alkali-isomerized linoleic acid. The method has also been used by REBELLO and DAUBERT (1951) to isolate methyl isolinoleate in 95·8 per cent purity from the hydrogenation products of methyl linolenate and by VON MIKUSCH (VON MIKUSCH, 1950; 1951) to separate isolinoleic acid (*trans*-10-*trans*-12-linoleic) from alkali isomerized or dehydrated castor oil. In

addition, it has been employed by JACKSON *et al.* (1952) for preparing purified linolelaidic acid (*trans*-9-*trans*-12-) as well as some other compounds which appeared to be *cis*-9-*trans*-12-, *cis*-9-*trans*-11-, *trans*-9-*trans*-11-, and *trans*-10-*cis*-12- octadecadienoic acids.

Repeated crystallization from petroleum ether at 2° to 3° was used by KHAN (1951) to purify stearolic acid. A similar technique was used in the purification both of stearolamide and of stearolyl alcohol. Ethanol (80 per cent) was used as solvent for the crystallization of 9,10-diketostearic acid. Deuterated methyl stearolate (methyl-9,10-dideutero oleate) was purified by crystallization from ether at — 41°.

Low temperature crystallization has also been used to purify other fatty acid derivatives such as hydroxy acids (ROE *et al.*, 1947) and fatty acid amides (ROE *et al.*, 1952).

INOUE and his co-workers in Japan have developed a method for separating fatty acids by means of their hydroxamic acids (INOUE and YUKAWA, 1940). The hydroxamic acids are crystalline substances with melting points considerably higher than those of the corresponding carboxylic acids. The hydroxamic acid derived from oleic acid, for example, melts at 61°. Fatty acids are converted quantitatively to hydroxamic acids by reaction with hydroxylamine under anhydrous conditions; the original acids can be later regenerated by refluxing with dilute alcoholic sulphuric acid solution.

Treatment of hydroxamic acids at 0° with organic solvents, such as alcohol, ether, petroleum ether, or carbon tetrachloride, is recommended for separating fatty acid mixtures into saturated and unsaturated fractions. The hydroxamic acid method is also reported to be superior to steam distillation as a means for separating volatile and non-volatile fatty acids (INOUE *et al.*, 1941).

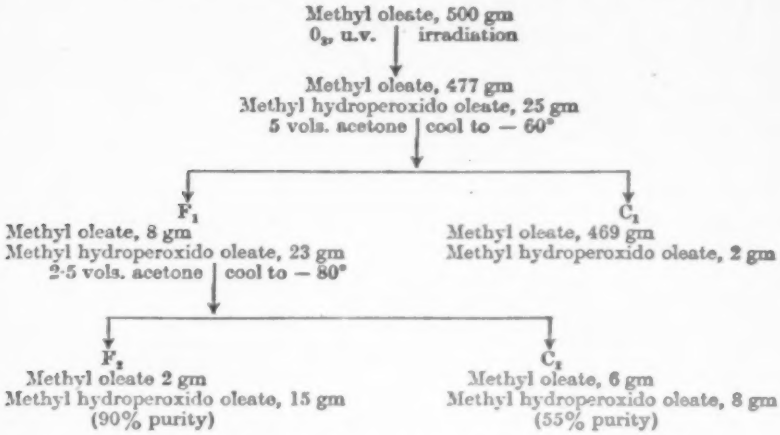
The technique has been suggested as a means for obtaining pure oleic acid from olive oil (INOUE and YUKAWA, 1941a) and pure erucic acid from rapeseed oil (YUKAWA and INOUE, 1942). Moreover, it is claimed that repeated crystallization of hydroxamic acids from organic solvents facilitates the preparation of highly purified linoleic acid from cottonseed oil and linolenic acid from soya bean oil (INOUE and YUKAWA, 1941b).

Another unusual fatty acid derivative which has been isolated by crystallization is methyl hydroperoxido oleate (SWIFT *et al.*, 1946). Methyl oleate was oxidized under u.v. radiation to yield about 5 per cent of the hydroperoxide. Separation was then effected by crystallization from acetone according to the scheme shown on page 89:

(*c*). *Pseudolipids*

Several interesting applications of the low temperature technique to the resolution of certain lipids which occur naturally but which are neither fatty acids nor their compounds and which have been designated as pseudolipids (BROWN, 1950) have been reported. One such application is that of removal of unsaponifiables from oils, as for example the sterols from peanut oil (OLIVER *et al.*, 1944) and from pike liver oil (SHANTZ, 1948) and the tocopherol

CHART 8
ISOLATION OF METHYL HYDROPEROXIDO OLEATE



fraction from cottonseed oil (SINGLETON and BAILEY, 1944). Crystallization from acetone at — 10° was applied to a tocopherol fraction of alkali-refined soybean oil to free it from steroids (STERN *et al.*, 1947). The unsaponifiable material from the liver oil of the seven-gilled shark (KARNOVSKY *et al.*, 1948) was separated into "solids" and "liquids" by crystallization at low temperatures from methanol and petroleum ether.

Pure synthetic Vitamin A aldehyde (ARENS and VAN DORP, 1949) was prepared by crystallization from petroleum ether at — 40° as was the phenyl azobenzoate ester of Vitamin A_2 alcohol (SHANTZ, 1948) from a higher boiling hydrocarbon solvent at — 30°.

(d) Miscellaneous applications to the separation of organic compounds

Although this review is primarily directed towards describing the uses of low temperature crystallization in the separation of lipids, a brief discussion of its application to organic compounds in general seems appropriate.

The organic chemist frequently uses crystallization as a method for purifying solid compounds, but he does not usually think of this technique in purifying substances which are liquids at room temperature. Separations of liquids which may be difficult by distillation can often be accomplished by crystallization at low temperature, and since the technique is simple, requires no complicated equipment, and is without hazard of thermal decomposition, the organic chemist might well consider a trial of the method. It is our opinion that the procedure is widely applicable in the separation of organic compounds.

In 1910 FISCHER (1910) cooled a mixture of nitrotoluenes (60 per cent o-, 30 per cent p-, 2 per cent m-) to — 18°, thereby causing the para isomer to crystallize out of solution. He also separated p-xylene from a xylene mixture by cooling with liquid air. This latter process has recently been applied on a commercial scale to the separation of xylene mixtures. By cooling such a

mixture to — 95°F, there can be separated crystals containing 75 to 85 per cent p-xylene. Recrystallization yields a p-xylene product of 95 per cent purity (*Chem. Eng.*, **59**, 219, 1952). Interest in this process was stimulated by the demand for p-xylene as a raw material in Dacron manufacture.

β-Ethyl naphthalene (SWIETOSLAWSKI, 1947) has been separated from an α-β-mixture by dissolving the mixture in an alcohol, such as methanol, and cooling it to — 40° to — 45°. At this temperature crystals of β-ethyl naphthalene separate from the solution and can be filtered off. Crystallization at low temperatures has also been employed in the purification of styrene (WEIR, 1946), durene (SCHMIDL, 1951), and chrysene (CURTIS, 1947).

Separations of m- and p-ethyl phenols (CISLAK and OTTO, 1949) was effected by virtue of the fact that the meta isomer forms with o-toluidine an addition compound which can be crystallized from solution at — 15°. The addition compound has use in fungicidal preparations, and m-ethyl phenol can be recovered from it in an essentially pure state. Mixtures of m- and p-cresol (ENGEL, 1945) may be resolved by a process comprising solution in a paraffinic hydrocarbon solvent and cooling to 0°. Crystals of p-cresol can then be removed by filtration. Washing these crystals and distilling off the last traces of solvent results in a product of 98·6 per cent purity. Crystalline 2,3,5-trimethyl phenol (LUTEN, 1944) may be separated from crude alkyl phenol mixtures by fractional distillation and subsequent crystallization at 0°. The deposited crystals of 2,3,5-trimethyl phenol are then recrystallized and may be used to produce trimethyl hydroquinone—an intermediate in Vitamin E synthesis.

Benzene, which contains 2 to 3 per cent of close-boiling hydrocarbon impurities, can be purified by crystallization from chilled liquefied butane (KEELING, 1949). About 50 per cent of the benzene can be recovered in this way, and the remainder can be subjected to azeotropic distillation with isopropyl alcohol.

Hydrocarbon mixtures which do not yield to separation by distillation can often be resolved by preferential crystallization. For example, mixtures of C_{12} to C_{18} hydrocarbons were separated into n-paraffin, iso-paraffin, and naphthenic groups by fractional crystallization with progressive cooling in an organic solvent (e.g. a mixture of acetone and dichloroethane (Compagnie Francaise de Roffinage, Fr. 1,012,345, 1952)). Low temperature crystallization has various applications in the petroleum industry for separating oil fractions with special drying (SCHNEIDER, 1950) or viscosity index-improving (STEWART and SCHAERER, 1944) properties; and crystallization of petroleum fractions from liquid propane at temperatures in the region of — 40° is used commercially for deasphalting and dewaxing (BRAY, 1938).

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4

SYNTHETIC DETERGENTS

W. Baird

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4

SYNTHETIC DETERGENTS

*W. Baird**

I. Introduction

Soap, in one form or another, has been known from very early times and its valuable cleansing, wetting, emulsifying and dispersing properties naturally led to its use in textile treatment processes such as dyeing. The rapid development of the synthetic dyestuffs industry in the nineteenth century focused attention on the weaknesses of soap, namely its instability to hard water and acids, and the need for a product possessing the valuable properties of soap without its defects became increasingly evident during this period. The first step towards such a product was attained about 100 years ago by treatment of vegetable oils such as olive and castor oils with sulphuric acid. These so-called sulphonated oils, for example Turkey Red Oil, found widespread use in the textile industry though their actual cleansing power was relatively poor. There was then a strange lapse of time before the recognition of the technical possibilities of manufacturing synthetic cleansing agents free from the disadvantages of soap and it was not until the First World War that the acute scarcity of natural fats and oils led the Germans to devote some attention to these possibilities. The first fruit of their researches was a di-*iso*-propylated naphthalene sulphonic acid marketed as *Nekal A* by the *Badische Anilin-und Soda-Fabrik A.G.* but again this agent was of value more for its wetting and emulsifying properties than for detergency. The first really efficient synthetic detergents appeared a few years later but were based on naturally occurring fats and oils. These were the *Gardinols* of *Böhme Fettchemie* and the *Igepons A* and *T* of the *I.G.* They have the structure

$$ROSO_3Na,\ RCOOC_2H_4SO_3Na \text{ and } RCON(Me)C_2H_4SO_3Na$$

respectively where R—O— is derived from a long chain fatty alcohol and RCO— denotes an oleic acid residue. Their chemical similarity to the fatty acid soaps, RCO_2Na, is obvious.

The inter-war years saw intense activity in the whole field of surface active agents stimulated, no doubt, by the drive for economic self-sufficiency in Germany and by the technical and commercial potentialities in other countries. The original market was in the industrial field where technical advantages could be more easily demonstrated but the economically more lucrative household market was attacked, principally by the *Proctor and Gamble Co.* with a

* The author is indebted to *Imperial Chemical Industries Ltd.*, Dyestuffs Division, for facilities which made possible this contribution.

fatty alcohol sulphate marketed as *Dreft*. The years of the Second World War and post-war years have seen enormous advances in the use of synthetic detergents in both the industrial and household markets due largely to advances in chemical technology rather than to new chemical discoveries, and to the scarcity and high cost of animal and vegetable oils and fats as well as to the merits of the products. To-day, synthetic detergents can be regarded as an important industry and production in the U.S.A. now surpasses that of soap.

It is not possible to draw any chemical line between products which have good detergent properties and closely related products which have poor detergent properties but which may be of technical value for other purposes, for example wetting. Detergency is frequently claimed along with other surface-active properties in the patent literature and standards of evaluation are rarely given. In any case, it must be understood that the properties of any given detergent are related to the conditions of use so that product A may be better than product B for washing woollens while the reverse may hold for cotton and in fact the same differences may hold for cotton under different conditions of soiling and washing. Further, few of the products under consideration are simple chemical entities and properties depend on the various components present in the starting materials as well as on the various inorganic salts etc. which may be present either adventitiously or purposely in the marketed products. An effort has therefore been made to give as comprehensive a picture as possible but with special emphasis on products of technical importance as detergents and where comparisons of detergency are given the limitations of such statements should be fully appreciated.

It is not possible to deal here with the physical-chemical aspects of detergency or with methods of evaluation. Suffice it to say that though the fundamental factors affecting detergency are known, their relative importance is not known and physical-chemical tests cannot be used to predict detergent properties. Methods of evaluation for the most part are efforts to duplicate in the laboratory, as far as possible, the particular conditions expected in use, and large-scale trials under practical conditions provide the final answer on performance. Other technological and psychological factors such as hygroscopicity, economics, physical form and foaming properties must also be taken into account for commercial reasons. For information on other surface-active agents, physical-chemical considerations and methods of evaluation the reader is referred to other sources.[1], [2], [3], [4]

The chemistry of synthetic detergents is essentially the solubilising of a hydrophobic hydrocarbon residue containing about 12 to 20 carbon atoms by means of a suitable hydrophilic group and the balance between these two governs in a rather general way the surface-active properties of the product. Solubilisation may be effected so that the hydrophobic part of the molecule forms part of the anion (anionic agents) or part of the cation (cationic agents) or it may be effected by means of groups which do not ionize in water (non-ionic agents): the subject-matter has been treated under these three headings. Since part of the object of research in this field has been to exploit cheap or

available sources of raw materials especially the natural fatty esters and petroleum-type hydrocarbons, an effort has been made to indicate the diverse ways the various raw materials can be used. The more important routes are indicated in the diagram below. In addition, a little consideration has been given to detergent adjuvants which play an important part in the commercial

ROUTES TO SYNTHETIC DETERGENTS

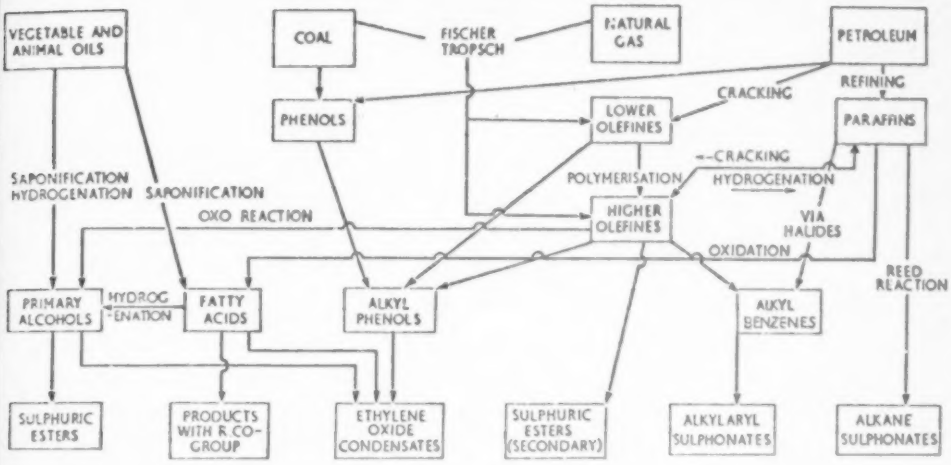


Fig. 1

success of the synthetic detergents. The subject-matter has therefore been dealt with under the main headings:

II. Anionic detergents.
  A. Products derived from natural fatty esters.
  B. Products derived from petroleum and related raw materials.
III. Cationic detergents from the above sources.
IV. Non-ionic detergents from the above sources.
V. Detergents from miscellaneous raw materials.
VI. Detergent adjuvants.

Suitable sub-headings have been employed according to the complexity of the subject-matter.

II. Anionic Detergents

The principal means of obtaining anionic types is by introduction of sulphonic acid groups $-\overset{|}{\underset{|}{C}}-SO_3Na$, sulphuric ester groups $-\overset{|}{\underset{|}{C}}-O-SO_3Na$ and, but less important, carboxylic acid groups $-\overset{|}{\underset{|}{C}}-CO_2Na$. Various miscellaneous acidic groups have also been used. The main chapters devoted to the production of

detergents from vegetable and mineral oils and the like are therefore restricted to these three solubilising groups whilst the use of the miscellaneous anionic solubilising groups with these starting materials is dealt with in a later chapter (*C*).

The solubilising groups can be introduced directly or attached by indirect means. The principal methods of making sulphonic acids are, of course, well known in chemistry and comprise mainly treatment with sulphuric acid or other sulphonating agent, addition of bisulphite to double bonds, replacement of halogen by reaction with sodium sulphite and so on. The sulphuric ester groups are introduced by the sulphation of alcoholic groups and by addition of sulphuric acid at double bonds. Much of the research has therefore been directed to producing from the various sources intermediates on which such simple reactions can be effected. The alternative method of reacting a suitable hydrophobic intermediate with an intermediate already carrying the solubilising group, again by methods quite well known to the organic chemist, has also been studied extensively and is particularly important for utilising the fatty acids.

A. Products derived from natural fatty esters

These raw materials provide sources of fatty acids and fatty alcohols within the C_{12} to C_{18} chain length range which experience has shown to be necessary for optimum detergency. It is of course true that the fatty acids provide the main technical source of the alcohols but it is convenient to consider these under separate headings. It has already been indicated that the solubilising group may be attached by one of a variety of linkages to the hydrophobic residue and for convenience in discussing the subject-matter many of these have been treated individually. The subject-matter has therefore been divided into the following sub-headings:

A.1. Products derived from fatty alcohols (sulphates, sulphonates and carboxylic acid salts).

(*a*) Production of fatty alcohols.
(*b*) Sulphated fatty alcohols.
(*c*) Ester derivatives of fatty alcohols.
(*d*) Ether derivatives of fatty alcohols.
(*e*) Other methods from fatty alcohols.

A.2. Products derived from fatty acids (sulphates, sulphonates and carboxylic acid salts).

(*a*) Ester derivatives of fatty acids: sulphates.
(*b*) Ester derivatives of fatty acids: sulphonates.
(*c*) Amide derivatives of fatty acids: sulphates.
(*d*) Amide derivatives of fatty alcohols: sulphonates.
(*e*) Amide derivatives of fatty acids: carboxylic acids.
(*f*) Derivatives of fatty amines.
(*g*) Miscellaneous fatty acid derivatives.

1. ***Products derived from fatty alcohols (sulphates, sulphonates and carboxylic acid salts).***

(a) Production of fatty alcohols. Spermacetti and sperm oil from the sperm whale are the principal natural sources of fatty alcohols. The former, for simplicity, can be regarded as a crude cetyl palmitate and the latter as crude oleyl oleate though other saturated and unsaturated esters of the same type are present. Saponification is a simple means of obtaining the required alcohols which can be separated from the by-product soap by super-heated steam distillation. The saponification process has been operated in a continuous manner on the large scale by the *I.G.*[5]

The technical development of catalytic hydrogenation processes has played a part in the extensive use of fatty alcohol derivatives as detergents. The normal procedure is to hydrogenate the glyceride or other suitable ester over a copper-containing catalyst at 240 to 270°C and 200 atm pressure and details of the *I.G.* technique have been given.[6] Under these conditions unsaturated esters are largely converted to the corresponding saturated alcohol and though special techniques such as using zinc-vanadium or cadmium-vanadium catalysts have been claimed[7] to give good yields of the unsaturated alcohols, these do not appear to have achieved technical success. An interesting departure from the normal catalytic method is by reduction of selected metal soaps[8] such as lead oleate.

The sodium-alcohol method of reducing esters to alcohols discovered by Bouveault and Blanc does not affect olefinic bonds and has been used extensively for producing unsaturated (and also saturated) alcohols in Germany and the U.S.A. *Deutsche Hydrierwerke* made their oleyl alcohol from sperm oil in this way, the obvious advantage over the saponification route being that both the alcohol and the acid residues of the oil are made available as alcohols: the *I.G.* method from butyl oleate has also been described.[9] Large-scale development of the Bouveault-Blanc method in the U.S.A. has been pioneered by the *duPont Company*. The chemistry of the reaction has been studied[10] and secondary alcohols such as methyl isobutyl carbinol found best as the reducing alcohol. An interesting account has been given of the process as used by the *Proctor and Gamble Co.*[11]

(b) Sulphated fatty alcohols. Sulphation is of course the cheapest possible method of solubilising fatty alcohols and involves treating the alcohol with, for example, sulphuric acid:

$$ROH + H_2SO_4 \rightarrow ROSO_3H + H_2O$$

It is interesting to record that cetyl alcohol was first sulphated by Dumas and Peligot in 1836, about 90 years before technical development of the sulphated fatty alcohols occurred. The earlier patent literature provides many references to methods of sulphating the fatty alcohols. Use of the normal sulphating agents such as sulphuric acid, oleum and chlorsulphonic acid was followed by the use of solvents for the reaction and the use of more selective sulphating agents. This was probably due to the need to effect the reaction

under as mild conditions as possible to avoid charring and undesirable side reactions. In the absence of a solvent the minimum temperature of sulphation is dependent on the melting point of the fatty alcohol which is about 48°C in the case of cetyl and lower with the lower and the unsaturated alcohols. The use of sulphur trioxide with sulphur dioxide as reaction medium under adiabatic conditions has been described recently.[2c5]

The unsaturated alcohols present a special problem in that in addition to sulphation of the alcohol group sulphation can also occur at the double bond:

$$—CH = CH— + H_2SO_4 \rightarrow —CH_2—CH(OSO_3H)—$$

Sulphation at the double bond instead of, or in addition to, sulphation of the hydroxyl group results in inferior detergent properties. Both reactions occur at normal temperatures with sulphuric acid and the former is less dependent on temperature than the latter. Patents have therefore appeared for sulphating with sulphur trioxide complexes such as those with pyridine and dioxan, sulphating with oleum, chlorsulphonic acid and pyrosulphates in the presence of bases such as pyridine, and so on.

In industrial practice, sulphuric acid and chlorsulphonic acid with or without solvents give acceptable results. Thus the *I.G.* sulphated lauryl* and oleyl alcohols with chlorsulphonic acid in the presence of chloroform: they also used a chlorsulphonic acid-urea-formamide mixture for sulphating oleyl alcohol.[12] HENKEL sulphated lauryl alcohol directly with chlorsulphonic acid at 25 to 30° and higher saturated alcohols at up to 45°C but the batch size was limited. For textile purposes, oleyl alcohol was sulphated with chlorsulphonic acid in pyridine to minimize attack at the double bond which is stated to result in increased substantivity to wool. For other general purposes HENKEL used 97 to 98 per cent sulphuric acid for both saturated and unsaturated alcohols: free sulphur trioxide causes discoloration with unsaturated alcohols.[5] Sulphuric acid must of course be used in excess of theory and products so made usually contain the sodium sulphate as diluent.

The extensive usage of sulphated alcohols has led to the development of continuous sulphation processes. This can be done by allowing acid and alcohol to flow together without temperature control and then neutralizing rapidly to minimize side reactions. Descriptions of the HENKEL procedure have been given.[6], [13] Alcohol and acid flow together from concentric feed lines on to a rotating disc which has two perforated flanges perpendicular to the plane of the disc. The mixture passes through these perforations and is thrown on to the walls of a pan where it collects in an annular trough and is cooled by external cooling to 35 to 45°C. The mixture flows over into a further pan where it is held to complete the sulphation and is then continuously neutralised, using a mixture of pre-neutralised alcohol sulphate and caustic alkali to facilitate

* Although not strictly correct, the terms lauryl, stearyl, oleyl etc. are used here to denote the alkyl group corresponding to the acid from which it is derived. This is convenient and avoids cumbersome terminology such as *cis*-$\triangle$9-octadecenyl for oleyl. The acyl group is denoted by stearoyl or other unambiguous term. In any case, a pure substance meriting a truly chemical name is rarely under consideration.

temperature control. Sulphation can also be effected continuously by spraying together the alcohol and chlorsulphonic acid.[207]

It is clear that although sulphation is an apparently simple process close attention to technical detail is essential for optimum results.

The properties of the sulphated alcohols—and this is general for all types of detergents—depend on the size of the hydrophobic residue used. Solubility decreases with increase in chain length so that sodium stearyl sulphate can only be used where temperatures approaching 100°C can be tolerated as in the case of cotton. The unsaturated alcohols are appreciably more soluble and sodium oleyl sulphate, like the lower saturated alcohol sulphates, can be used for woollens which are washed at a lower temperature. Apart from the limitations imposed by solubility, the higher members of the series give better detergency than the lower members of the series. It should be borne in mind, however, that pure alcohols are not used in practice, and solubility and detergent properties will be affected by the particular mixture of alcohols used. It seems to be generally accepted that for light duty detergency in the home, i.e. for washing woollens and fine fabrics, sodium lauryl sulphate is the most suitable on account of its ready solubility and excellent foaming power. The alcohols for this purpose are based on such raw materials as coconut and palm kernel oils. For industrial use, sodium oleyl sulphate is preferred because of its better detergency: difficulties with respect to physical form militate against its use in the household market. Sulphated alcohols in general are stable to alkali but hydrolyse under acid conditions. In common with all anionic agents they are substantive to animal fibres and the resultant "handle" conferred on wool is considered attractive by some users.

The properties of the technical products are also affected by the purity of the alcohols used and by the sulphation conditions. Thus, paraffinic hydrocarbons may be present as impurities in alcohols obtained by catalytic hydrogenation and unsulphated alcohols may remain in the final product. Generally, such impurities are in sufficiently small amount not to be detrimental and in fact may even be advantageous in the case of unsulphated alcohol which can have a favourable influence on foaming properties. For special purposes, e.g. cosmetics, impurities can be removed by relatively simple extraction processes.

The sodium salts are, of course, the most extensively used but other salts, especially the triethanolamine salts, find outlets where special properties are desired, for example greater water solubility for liquid shampoos. They are marketed under a great variety of trade names.

(c) *Ester derivatives of fatty alcohols.* Fatty alcohol esters carrying sulphonic acid, sulphuric ester or carboxylic acid groups to confer solubility are quite readily prepared.

Direct esterification of the alcohol with sulphoacetic acid[14] or with chloracetic acid followed by reaction with sodium sulphite gives products of the type

$$R—O—OC—CH_2—SO_3Na$$

Related products can be obtained by addition of bisulphite to esters of unsaturated acids such as acrylic and crotonic acid:

$$R—O—CO—CH=CH_2 + NaHSO_3 \rightarrow RO—CO—CH_2CH_2SO_3Na$$

Maleic mono- and di-esters, giving rise to sulphosuccinic esters by addition of bisulphite,[15] have been carefully studied and products of the Aerosol type

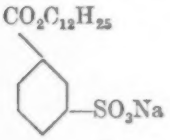
$$\begin{array}{l} NaO_3S—CH—CO_2R \\ \quad\quad\quad\quad | \\ \quad\quad\quad CH_2—CO_2R^1 \end{array}$$

have been found of considerable technical interest but mainly as wetting agents where R and R^1 are, for example, 2-ethylhexyl or lower alkyl groups. More complex unsaturated acids, for example Diels-Alder type adducts of maleic anhydride, have been used in place of maleic acid.

An alternative procedure is to convert the alcohol to a hydroxy acid ester, e.g. with lactic acid followed by sulphation of the hydroxy group[16]:

$$ROH \rightarrow R—O—CO—CH(OH)CH_3 \rightarrow R—O—CO—CH(OSO_3Na)CH_3$$

Aromatic residues can also be introduced by making esters, e.g. of m-sulphobenzoic acid[17] (I), sulphophthalic acid[18] (II) or phenoxyacetic acid followed by sulphonation[19] (III).



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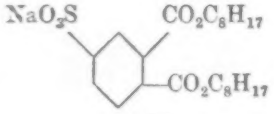

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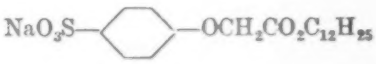


III

Products having the carboxylic ester group are of course subject to hydrolysis especially under alkaline conditions and this, together with the extra cost involved as compared with direct sulphation of the alcohols, limits their usefulness. Nevertheless, the lauryl sulphoacetate and sulphobenzoate find commercial outlets for special purposes, e.g. in dentifrices where taste is a point of importance. A route to the related sulphosalicylic esters has been described recently.[20a]

The higher alcohols have been reacted with anhydrides[20] such as phthalic, maleic and succinic giving rise to products of the type ROCO—X—CO_2Na, but although these are claimed to have cleansing properties they do not appear to have found commercial outlets presumably on grounds of solubility or lack of resistance to hard water.

Carbamic ester linkages have been used[21] in place of carboxy ester groups to introduce the solubilising groups, e.g.:

$$ROH \xrightarrow{COCl_2} ROCOCl: \qquad ROCOCl + NH(Me)C_2H_4SO_3Na \rightarrow ROCON(Me)C_2H_4SO_3Na$$

$$ROCOCl + NH(Me)CH_2CO_2Na \rightarrow ROCON(Me)CH_2CO_2Na$$

$$ROCOCl + NH_2C_2H_4OH \rightarrow ROCONHC_2H_4OH \rightarrow ROCONHC_2H_4OSO_3Na$$

which bear a close structural resemblance to *Igepon T*, *Medialan A* and *Igepon B* respectively (see pp. 110, 113).

Fatty esters of amino acids,[22] mercapto acids[23] etc. have been used for attaching the solubilising group via the amino and mercapto residues, e.g.:

$$ROCOC_2H_4NH_2 + HO_2CC_2H_4CO_2H \rightarrow ROCOC_2H_4NHCOC_2H_4CO_2H$$

$$ROCOCH_2SH + ClC_2H_4SO_3Na \rightarrow ROCOCH_2SC_2H_4SO_3Na$$

(*d*) *Ether derivatives of fatty alcohols.* Numerous ether derivatives can be prepared by standard methods, e.g.:

$$ROH + \begin{matrix} CH{-}CO_2Et \\ \| \\ CH{-}CO_2Et \end{matrix} \rightarrow \begin{matrix} CH_2CO_2Et \\ | \\ RO{-}CH{-}CO_2Et \end{matrix} \rightarrow \begin{matrix} CH_2CO_2Na \\ | \\ RO{-}CH{-}CO_2Na \end{matrix}$$ [24]

$$ROH \rightarrow RO \cdot C_2H_4Cl \rightarrow ROC_2H_4SO_3Na$$ [25]

$$ROH \rightarrow ROC_2H_4OC_2H_4Cl \rightarrow ROC_2H_4OC_2H_4SO_3Na$$ [26]

$$ROH \rightarrow RCl \rightarrow RO\text{—}C_6H_4\text{—}R\text{—}O\text{—}C_6H_4\text{—}SO_3Na$$ [27]

$$ROH \rightarrow ROCH_2\text{—}C_6H_4\text{—}R\text{—}O\text{—}CH_2\text{—}C_6H_4\text{—}SO_3Na$$ [28]

but the most practicable is by direct reaction with an alkylene oxide, e.g. ethylene oxide or cyclohexene oxide to give hydroxy ethers which are sulphated[29]:

$$ROH + n\,CH_2\text{—}O\text{—}CH_2 \rightarrow RO(C_2H_4O)_nH \rightarrow RO(C_2H_4O)_n\text{—}SO_3Na$$

Chlormethyl ethers readily made from the alcohol, formaldehyde and hydrochloric acid, can be reacted with ethylene oxide and then with sodium sulphite[13]:

$$ROCH_2Cl \rightarrow ROCH_2OC_2H_4Cl \rightarrow ROCH_2OC_2H_4SO_3Na$$

(*e*) *Other methods from fatty alcohols.* Many other reactions available to the organic chemist have been examined for converting fatty alcohols to surface-active compounds but apparently have not achieved commercial success for economic or technical reasons. Thus, the alcohols can be condensed with aromatic hydrocarbons and phenols to give alkylaromatics and alkyl phenols respectively: these can be sulphonated etc. They can be dehydrated to olefines and the olefines converted to alkylaromatic types. The olefines can also be used in a variety of other ways, e.g. sulphated, sulphonated, reacted with hypochlorous acid and the resultant chlorhydrin reacted with sodium sulphite, and so on.

The n-alkyl halides have also been used extensively as intermediates giving, in addition to the above types of compounds, mercaptans which can be oxidised to sulphonic acids or converted by standard procedures to many of the thio,

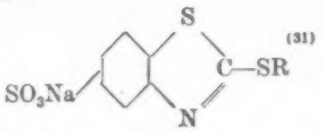
sulphoxide or sulphone products corresponding to the O-ether derivatives described above, e.g.:

$$ROH \rightarrow RCl \rightarrow RSH \rightarrow RSCH_2CH_2OH \rightarrow RSCH_2CH_2OSO_3Na.^{(30)}$$

$$\searrow$$

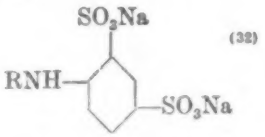
$$RSO_2CH_2CH_2OH \rightarrow RSO_2CH_2CH_2OSO_3Na$$

Thiazole derivatives such as



(31)

and amino derivatives such as

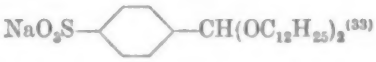


(32)

have also been made by means of the alkyl halide.

In general, however, alkylaromatic, olefine and mercaptan derivatives are much more economically available from petroleum-type sources and will be discussed in greater detail later.

Other alcohol derivatives described include glucosides and acetals carrying sulphonic acid or sulphuric ester groups, e.g. the sulphobenzaldehyde acetal of lauryl alcohol,



(33)

The C_8—C_{10} alcohols are also available from the lower fractions of coconut oil alcohols. These are of too short chain length to be used as such (unless two are used) but they can be converted to detergent intermediates by means of the Guerbet reaction which may be summarised as

$$RCH_2CH_2OH + R'CH_2OH \xrightarrow{Na} R—\underset{|}{\overset{CH_2OH}{CH}}—CH_2R'$$

These alcohols are therefore branched chain primary alcohols and resemble in structure the *Oxo* alcohols which are described later. A review of the chemistry of the reaction and the properties of the products is given by MACHEMER.[34] The more obvious objection to the process is one of economics though 2-hexyldecanol is stated to be produced from n-octyl alcohol and the sulphate is said to be similar in detergent properties to the sulphates from myristyl and cetyl alcohols.

2. *Fatty acid derivatives (sulphates, sulphonates and carboxylic acid salts).*

Much ingenuity and effort have been expended in obtaining useful products from the fatty acids which are cheap and plentifully available in normal times.

In times of scarcity conversion to synthetic detergents results in a more efficient usage as against their direct use as soaps, apart from questions of use in hard water.

(*a*) *Ester derivatives of fatty acids: sulphates.* Conversion of fatty acids or glycerides to hydroxyalkyl esters followed by sulphation to products such as

$$RCO_2CH_2CH_2OSO_3Na$$

is one of the simplest and cheapest methods which can be devised for utilising the fatty acids and it has been intensively studied. The monoglycerides are an obvious practical answer: they can be made either by alcoholysis of a naturally occurring glyceride with glycerol or by esterifying a fatty acid from any source with an excess of glycerol. Both these procedures give mixtures of the various glycerol esters, the relative yield of each depending on the conditions used. The monoglycerides can be isolated if desired by suitable distillation techniques. The monoglycerides can be sulphated by the methods already described for the fatty alcohols. The finished products contain both the carboxylic ester and the sulphuric ester group and are therefore susceptible to hydrolysis under both acid and alkaline conditions. This can give rise to difficulties in producing material of uniform quality and processes have been designed to overcome these. Thus, the crude sulphation product may be added to a suspension of a neutralising agent such as sodium carbonate in an inert organic solvent for the sulphation mass such as ethanol,[35] or the sulphuric ester may be neutralised by contacting it in an atomised state with a base such as ammonia in vapour form.[36]

The most interesting procedure technically for making sulphated monoglycerides comprises the direct reaction of the triglyceride with glycerol and sulphuric acid: oleum can also be used.[37] This process has been made continuous[38] and is obviously attractive economically. Glycerol trisulphuric ester can be used:

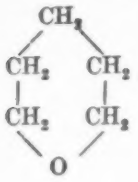
$$NaO_3SOCH_2CH(OSO_3Na)CH_2O(SO_3Na) + C_{11}H_{23}CO_2H \rightarrow C_{11}H_{23}CO_2CH_2CHOHCH_2OSO_3Na^{[39]}$$

Ethylene glycol, propylene glycols, polyglycols, pentaerythritol and the like have also been used as polyhydric alcohols in place of glycerol and the hydroxy esters have been made from the fatty acid soap and the requisite chlorhydrin, e.g. $Cl(CH_2)_6OH$. Products of analogous structure have been made by reacting a fatty acid with formaldehyde, ethylene glycol and sulphuric acid[40]:

$$RCO_2H + CH_2O + HOC_2H_4OH + H_2SO_4 \rightarrow R{-}CO_2{-}CH_2{-}O{-}CH_2CH_2{-}O{-}SO_3Na$$

Hydroxy esters can also be made by reacting a fatty acid with an epoxide and an interesting variation of this is to interact a polymethylene oxide with a fatty acid and a sulphonating agent thereby achieving the desired product in one stage. For example, tetrahydropyran, lauric acid and chlorsulphonic

acid are heated together until water soluble and the mass then neutralised. The reaction is represented thus:



$$+ RCO_2H + ClSO_3H \rightarrow RCO_2CH_2CH_2CH_2CH_2CH_2OSO_3H + HCl^{(13)}$$

Similar products are made by heating fatty esters of tetrahydrofurfuryl alcohol with sulphuric acid.[41]

Esters of unsaturated alcohols, e.g. allyl and methallyl alcohols, can also be made and sulphated at the double bond.[42]

As indicated before, products containing carboxylic ester groups are susceptible to hydrolysis and hence they are of limited value under alkaline conditions. Their use in practice is therefore somewhat restricted, but nevertheless the sulphated monoglyceride type has been developed extensively in the U.S., pioneered mainly by *Colgate-Palmolive-Peet* as *Vel* and the *Artic Syntex* products for domestic use: the ammonium salt is marketed for shampoos.

(*b*) *Ester derivatives of fatty acids: sulphonates.* The earliest and simplest detergent of this type to achieve technical importance was *Igepon A* (*I.G.*), the oleic ester of isethionic acid (β-hydroxyethane sulphonic acid):

$$C_{17}H_{33}COOCH_2CH_2SO_3Na$$

The procedure used by the *I.G.*, described by Brown,[43] involves heating the acid chloride with isethionic acid sodium salt at about 115°C in a specially designed reactor fitted with heavy duty mixing so that the initial pasty mass is converted to a granular powder as the reaction proceeds. It has also been made by reacting the fatty acid with sodium isethionate at 180 to 200° though this is believed to be a less desirable method.[19] The intermediate acid chloride is obtained by reaction of phosphorus trichloride on the acid and the sodium isethionate by reacting sodium bisulphite with ethylene oxide.

Alternative methods of preparation have been proposed, e.g.:

$$RCO_2Na + ClC_2H_4SO_3Na \rightarrow RCO_2C_2H_4SO_3Na$$

which is claimed to be effected best in the presence of a solvent such as a fatty acyl ethanolamide or formylmorpholine,[44] and by reacting chloroethyl esters with sodium sulphite:

$$RCO_2C_2H_4Cl + Na_2SO_3 \rightarrow RCO_2C_2H_4SO_3Na$$

Likewise sulphoalkyl esters other than the sulpho-ethyl have been made by similar means, e.g.:

$$RCO_2CH_2CHOHCH_2SO_3Na^{(45)}$$

$$RCO_2C_2H_4OC_2H_4SO_3Na^{(46)}$$

Esters carrying a tertiary carbon atom, e.g. isobutyl laurate, can be sulphonated with vigorous sulphonating agents[47]:

$$RCO_2CH_2CHMe_2 \rightarrow RCO_2CH_2C(Me_2)SO_3Na$$

More complex products can be obtained by making sulphoacetates of hydroxy alkyl esters or sulphoacyl derivatives of aminoalkyl esters, e.g.:

$$RCO_2C_2H_4OCOCH_2SO_3Na^{[48]} \text{ and } RCO_2C_2H_4NHCOCH_2SO_3Na^{[49]}$$

The latter, known as Emco type detergents, are stated to be highly effective in preventing lime soap deposits when incorporated in soap.[50] Fatty esters of unsaturated hydroxy esters (and amides) such as the lauric ester of allyl glycollate have been sulphonated* to give mixtures containing sulphonic acid and sulphuric ester groups[51]:

$$RCO_2CH_2CO_2CH_2{-}CH{=}CH_2 \rightarrow RCO_2CH_2CO_2CH_2CHOHCH_2SO_3Na + RCO_2CH_2CO_2CH_2CH(OSO_3Na)CH_2SO_3Na$$

Products containing aromatic residues, e.g.:

$$C_{12}H_{25}CO_2CH_2CONH{-}C_6H_4{-}SO_3Na$$

have been described.[52]

It will have been noted that two shorter chain alkyl groups can be used in place of one long chain alkyl group (p. 104) and this is further exemplified by using C_4—C_{10} di-esters of glycerol sodium sulphonate

$$CH_2OH.CHOH.CH_2SO_3Na^{[53]}$$

For high detergency with the *Igepon A* type product the *I.G.* preferred the oleic ester of sodium isethionate though for household use the product derived from coconut oil fatty acids gave better foaming. Products of this type, though good detergents in neutral solution, suffer from hydrolysis particularly in alkali and therefore find their main outlet in light duty detergency, e.g. for wool.

(*c*) *Amide derivatives of fatty acids: sulphates.* These may be regarded as of the general formula

$$R{-}CO{-}NH{-}X{-}OSO_3Na$$

The alkanolamines, and particularly ethanolamine[54], suggest themselves as convenient hydroxyamines and sulphated lauric ethanolamide,

$$C_{11}H_{23}CONHCH_2CH_2OSO_3Na$$

has been widely used in the U.S.A.

The fatty acyl ethanolamides can be made by heating the fatty acid and ethanolamine at about 170 to 180° but this gives rise to a product containing some of the β-aminoethyl ester as a by-product which detracts from the detergent and foaming properties of the final product and improved results are

* Although the action of sulphuric acid on double bonds gives rise to sulphuric esters under mild conditions, more vigorous conditions can give hydroxy (or sulphato) sulphonic acids.

obtained by acylating with the acid chloride. An alternative procedure is to react the glyceride directly with the alkanolamine at about 100°C, by-product glycerol being recovered by washing.

The sulphation of these alkanolamides can be done by the usual means of sulphuric acid or chlorsulphonic acid though special techniques have also been devised.[55] According to the *I.G.* procedure for *Igepon B*, the oleic amide of 1-amino-3-hydroxybutane,

$$C_{17}H_{33}CONH—CH_2—CH_2—CH(OH)—CH_3$$

was sulphated with chlorsulphonic acid in a urea-formamide mixture so as to avoid attack on the double bond.[13] An alternative method proposed for lauric ethanolamide is by the Schotten-Baumann reaction on the aminoethyl sulphuric ester:[57]

$$C_{11}H_{23}COCl + NH_2CH_2CH_2OSO_3Na \rightarrow C_{11}H_{23}CO.NH.CH_2.CH_2.OSO_3Na$$

According to SCHWARTZ and PERRY,[2] the properties of the sulphated ethanolamide of coconut fatty acids vary enormously with the degree of purity. Pure preparations are excellent foamers and detergents and are equal to sulphated alcohols but many of the commercial products contain sufficient unsulphated materials to detract markedly from their surface-active properties. It is known that they are subject to hydrolysis in the presence of water and difficulties with regard to purity may therefore well arise during the working up and drying of these products; they are stable when dry. More recent work has shown that in the particular case of sulphated stearethanolamide the presence of unsulphated ethanolamide is necessary to give optimum detergency for wool.[56] The *I.G.* considered that the isobutanolamide derivative is more stable to hydrolysis than the ethanolamide and that in the case of the ethanclamide, better stability is obtained by reacting with ethylene oxide to give a product of the type $RCONH(C_2H_4O)_nH$ before sulphating.[13], [58] In this event it would possibly be better to use stearic rather than lauric acid as the acylating agent to compensate for the solubilising action of the ethenoxy groups.

Hydroxy amines other than the above have of course been mentioned in the literature, e.g. diethanolamine, glycerolamine and trimethylolaminomethane.[59] Similarly, these other alkylolamides can be reacted with ethylene oxide and sulphated. Such variations do not appear to have achieved technical importance presumably mainly on economic grounds.

Products closely related to the above can be obtained by addition of sulphuric acid to unsaturated amides:[60]

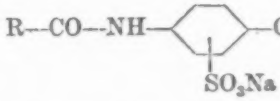
$$R—CO—NHCH_2CH = CH_2 + H_2SO_4 \rightarrow RCONHCH_2CH(OSO_3H)CH_3$$

(*d*) *Amide derivatives: sulphonates.* The original representative of this type of product to be marketed is *Igepon T* (*I.G.*):

$$R—CO—N(CH_3)C_2H_4SO_3Na \text{ (R—CO— = oleic acid residue)}$$

which is made by the normal Schotten-Baumann technique from the acid

chloride and N-methyltaurine under aqueous alkaline conditions.[43] The methyltaurine is produced from sodium isethionate and methylamine by reaction at 270 to 290°C and 200 atm, though other routes are available. This type of product has of course been extensively investigated using a wide variety of amino sulphonic acids, both aliphatic and aromatic. The *I.G.* preferred methyltaurine as an intermediate because of the excellent detergent and lime-soap dispersing properties of the acyl derivatives. The parent taurine, made from ammonia instead of the relatively expensive methylamine, gave a product somewhat inferior in detergent properties and, owing to formation of by-product ditaurine, $NH(C_2H_4SO_3Na)_2$, was obtained in poorer yield. An interesting account of work on *Igepon T* types is given by KASTENS and AYO.[61] Aromatic amino sulphonic acid derivatives have been examined in detail by VENKATARAMAN[62] and a product of this type, *Lissapol LS*, has been made by *I.C.I.*:



RCO = oleic acid residue

Products such as *Igepon T* have high stability to acid and alkali and have found extensive use as general purpose detergents in Germany and the U.S.A.: the *I.G.* considered them rather better detergents than the sulphated alcohols and the best all-purpose textile detergents. As in the case of the sulphated alcohols, products from a C_{18} saturated fatty chain are of too low solubility and the oleic derivative is preferred for industrial use for both cotton and woollens, while products from coconut or palm kernel acids are preferred for household purposes. The latter are, however, more hygroscopic than the corresponding alcohol sulphates and require special packaging. Such products have also been recommended extensively on account of their ability to disperse lime soaps. Recent work by KNOWLES *et al.*[63] suggests that laboratory tests on which general claims for lime soap dispersing properties have been based may be of little practical value and again emphasizes the need for trial under conditions of actual use. Much experience with the *Igepon T* types is of course available.

Analogues of *Igepon T* have been made by the route:

$$ClC_2H_4OC_2H_4Cl \xrightarrow{Na_2SO_3} ClC_2H_4OC_2H_4SO_3Na \xrightarrow{MeNH_2}$$

$$MeNHC_2H_4OC_2H_4SO_3Na \xrightarrow{RCOCl} RCON(Me)C_2H_4OC_2H_4SO_3Na^{[64]}$$

Acyl sulphamic acids of the type $RCONHSO_3Na$, made by sulphonating the amide or acylating sodium sulphamate have been described.[65] More recently the *I.G.* have been interested in products of the type $RCONHCH_2SO_3Na$ made by reaction of the fatty acid amide with formaldehyde-bisulphite using piperidine as a catalyst.[13] *Luwipal R* was made in this way from waste fatty acids but apparently it is not easy to effect complete reaction and properties were not very satisfactory.

Other aldehydes with bisulphite, including unsaturated aldehydes with two mols of bisulphite which gives rise to disulphonated products thus:

$$RCONH_2 + OCHCH{=}CH_2 + 2NaHSO_3 \rightarrow RCONH{-}\underset{\displaystyle SO_3Na}{\underset{|}{CH}}{-}CH(SO_3Na)CH_3$$ [66].

have been used in place of formaldehyde-bisulphite: sulphonated aldehydes can also be used.

The *I.G.* were also interested in products made by the route:

$$RCONH_2 \rightarrow RCONHCH_2Cl : RCONHCH_2Cl + \underset{\displaystyle O}{CH_2CH_2} \rightarrow$$
$$RCONHCH_2OC_2H_4Cl \rightarrow RCONHCH_2OC_2H_4SO_3Na$$ [13]

More complex structures in which formaldehyde is used to provide the connecting link between the fatty amide and the solubilising group have been made by means of Mannich-type reactions, e.g.:

$$RCONH_2 \rightarrow RCONHCH_2OH :$$
$$RCONHCH_2OH + HN(Me)C_2H_4SO_3Na \rightarrow RCONHCH_2N(Me)C_2H_4SO_3Na$$ [67]

$$RCONHCH_2OH + HSC_2H_4SO_3Na \rightarrow RCONHCH_2SC_2H_4SO_3Na$$ [68]

$$RCONH_2 + CH_2O + C_6H_4(OMe) \rightarrow RCONHCH_2{-}C_6H_3(OMe) \rightarrow$$
$$RCONHCH_2{-}C_6H_3(OMe)(SO_3Na)$$ [69]

Fatty arylamide derivatives with the sulphonate group attached indirectly to the aromatic residue are known, e.g.:

$$RCONH{-}C_6H_4{-}CON(Me)C_2H_4SO_3Na$$ [70]

or alternatively the acylamide residue may be attached indirectly as in

$$RCONHC_2H_4O{-}C_6H_4{-}SO_3Na$$ [71]

The ethanolamides again can give rise to sulphonic acid derivatives by means used for the fatty alcohols themselves and sulphoacetates of the type

$$R{-}CO{-}NHC_2H_4O_2CCH_2SO_3Na$$ [72]

are of commercial interest.

(e) *Amide derivatives: carboxylic acids.* Although the soap-forming fatty acids give insoluble calcium and magnesium salts, the introduction of suitable groups between the fatty residue and the carboxy group can result in a great improvement in solubility so that some of these can be used satisfactorily in all but very hard water. Various fatty acyl derivatives of amino acids such as

aspartic acid and N-β-hydroxyethylglycine have been examined. The first product of this type to be marketed, *Medialan A* (*I.G.*), is the oleic amide of sodium sarcoside,

$$C_{17}H_{33}—CO—N(Me)CH_2CO_2Na$$

Manufacture is by the normal Schotten-Baumann process;[43] the intermediate sarcosine can be made by the usual methods from methylamine, formaldehyde and hydrogen cyanide, or from methylamine and chloracetic acid.

Medialan A is stated to be specially useful in the milling of wool. Detergent properties are only of the same order as sodium oleate but its mild action on the skin coupled with moderate resistance to hard water make products of this type of interest for use on the human body. For such outlets, the coconut oil acid derivative is preferred on account of its superior foaming power but the physical properties of this are poor so that the *I.G.* were interested in using mixtures of the stearic and coconut oil derivatives for shaving preparations.[43]

Closely related to the *Medialans* are the *Lamepons* (Germany)[13] and *Maypons** (U.S.A.) obtained by using amino acids or the lower polypeptides instead of sarcosine, the intermediates being obtained by hydrolysis of proteins, e.g. leather scrap. Variations on this theme are obtained by reacting fatty acylethanolamides with chloracetic acid,[73] by benzylating the amino acids from proteins before acylating,[74] by esterifying the alkanolamides with e.g. diacetyltartaric acid,[75] and so on.

(*f*) *Derivatives of fatty amines.* Fatty amines are quite readily available from fatty acids by the route

$$RCO_2H \xrightarrow{NH_3} RCONH_2 \xrightarrow{-H_2O} RCN \xrightarrow{H} RCH_2NH_2^{(5)}$$

and can give rise to a host of derivatives some of which bear a formal resemblance to many of the fatty amide derivatives already described having the linkage RNH—CO— in place of RCO—NH—. Thus the sulphoacetyl derivative $RNHCOCH_2SO_3Na$[76] resembles the *Igepon T* type $RCONHC_2H_4SO_3Na$ while closely related to the *Aerosol* type (p. 104) are the products

$$C_{18}H_{37}NHCOCH_2\underset{\displaystyle SO_3Na}{\underset{|}{CH}}—CO_2Na^{(77)} \text{ and } C_{18}H_{37}N\underset{\displaystyle CO_2Na—CH_2—\underset{|}{CH}—CO_2Na}{\underset{|}{}}—CO—CH_2—\underset{\displaystyle SO_3Na}{\underset{|}{CH}}—CO_2Na^{(78)}$$

derived from maleic acid and octadecylamine by acylation and addition reactions followed by addition of bisulphite at the double bond. These are marketed as detergents under the names *Aerosol* 18 and *Aerosol* 22 respectively.

The simple amic acids such as

$$RNHCOCO_2H \text{ and } RN(Me)COCH_2OCH_2CO_2H$$

bear a resemblance to *Medialan A* above and many products of this type including those from aromatic acids have been examined, good detergency and resistance to hard water being claimed.[79] In general, however, the fatty

* A list of products marketed under trade names is given in Ref. 4: or see A. J. Hall, *Modern Textile Auxiliaries*, Thos. Skinner & Co., Manchester.

amines are relatively expensive intermediates thus limiting their commercial usefulness. Other methods can therefore be summarised schematically, interesting examples being:

$RNH_2 \rightarrow RNHC_2H_4OH \rightarrow RNHC_2H_4OSO_3Na$[80]

$RNH_2 \rightarrow RNHC_2H_4SO_3Na$[81]

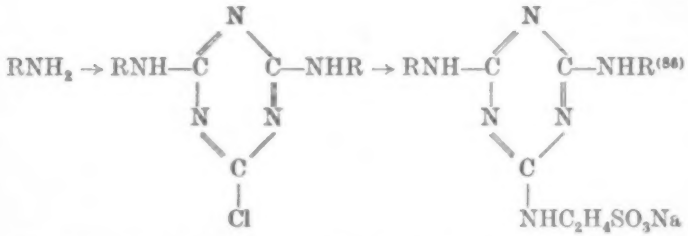
$RNH_2 \rightarrow RNHCOCH_2SH \rightarrow RNHCOCH_2SC_2H_4SO_3Na$[82]

$RNH_2 \rightarrow RNCO \rightarrow RNHCONHC_2H_4SO_3Na$[83]

$RNH_2 \rightarrow RNHCOCHOHCH_3 \rightarrow RNH.CO.CH(OSO_3Na)CH_3$[84]

$R(R')NH \rightarrow RN(R')COCl \rightarrow R(R')NCON(Me)CH_2CO_2Na$[85]

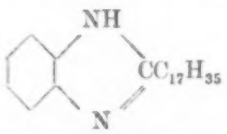
$RNH_2 + CH_2 = CHCO_2Me \rightarrow RNHCH_2CH_2CO_2Me \rightarrow RNHCH_2CH_2CO_2Na$[85]



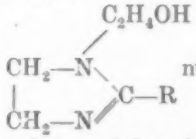
This last method, which depends on the stepwise reaction with the halogens in cyanuric chloride, is capable of many variations.

It should be appreciated that although all these amine derivatives are classified here as anionic, some of them contain a basic amino residue and are amphoteric in character: they are sometimes described as "ampholytic" agents.

(*g*) *Miscellaneous fatty acid derivatives.* Aromatic diamines can be used as intermediates giving iminazole derivatives. Thus 2-heptadecylbenziminazole,

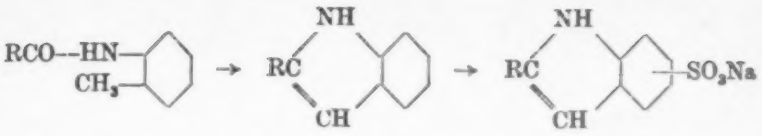


from stearic acid and o-phenylene diamine can be sulphonated to the monosulphonate (marketed as *Ultravon K*) or the disulphonate[87] (*Ultravon W*) which, unlike the monosulphonate, is soluble in acids: it is an effective detergent for wool. Hydroxyethyliminazolines,



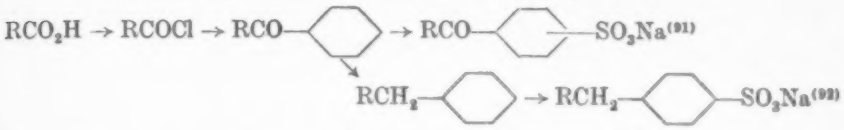
made from hydroxyethylethylenediamine and fatty acids, have been sulphated.[88]

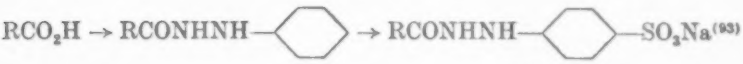
Indole derivatives can be made by ring closure of acylated o-toluidine and sulphonating:[89]

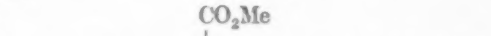


Other routes employing fatty acids are:

$$RCH_2CO_2H \rightarrow RCHBrCO_2H \rightarrow RCH(SO_3Na)CO_2Na^{(90)}$$







$$RCO_2H \rightarrow RCONHNH\text{—}C_6H_4\text{—}SO_3Na^{(93)}$$

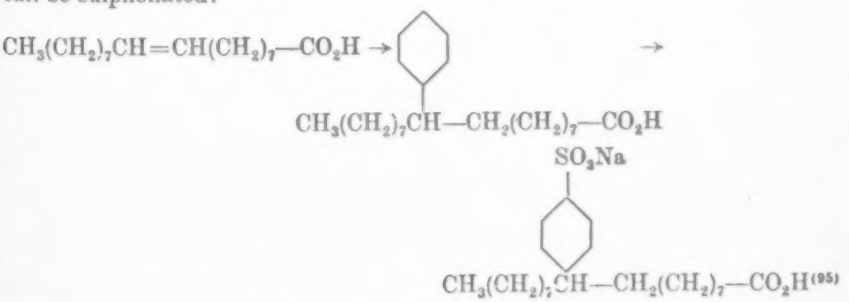
$$RCH_2CO_2H \rightarrow RCHBrCO_2H \rightarrow RCHBrCO_2Me \rightarrow RCH(CO_2Me)N(Me)CH_2CH_2SO_3Na^{(94)}$$

$RCO_2H \rightarrow RCO\text{—}NHC_2H_4NH_2 \rightarrow$ types of derivatives described under amines

$RCO_2H \rightarrow RCN \rightarrow RCSNH_2 \rightarrow$ analogues of the amide derivatives.

The acids can also be converted to: ketones which in turn are reduced to alcohols; into acyloins and glycol derivatives; into carbinols by means of Grignard reagents; to aldehydes which are converted to glucosides or hydroxy acetals; to partial esters of polyhydroxy acids, e.g. tartaric acid. All these intermediates are then sulphated.

In addition to these reactions which make use of the carboxy group or groups derived therefrom, many reactions have been based on the olefinic bond of unsaturated acid derivatives such as esters, including those available from natural sources, and amides. The principal reaction involved here is sulphation at the double bond of oleic acid derivatives and such products find extensive use in industry as wetting agents, dyeing assistants etc., but have little value as detergents. The double bond can also be used for other addition reactions to give intermediates for further reaction, for example condensation with aromatic hydrocarbons to give alkyl aromatic derivatives which can be sulphonated:



Isomers will, of course, be formed in such reactions.

Likewise, use has been made of the hydroxy group present in ricinoleic acid (from castor oil) and in the hydroxystearic acid obtained therefrom by hydrogenation. Side reactions complicate the chemistry of sulphating unsaturated and hydroxy esters, but they need not be discussed here.

B. Products derived from petroleum and related raw materials

Modern developments in the technology of petroleum, petroleum chemicals and related synthesis products from coal and natural gas have provided sources of intermediates which give rise to the major proportion of synthetic detergents now in commercial use. It is not possible here to give more than a brief outline of the processes involved in producing these intermediates but an extensive literature is available to those interested in pursuing this aspect. Broadly, it may be said that natural petroleum and synthesis products can give rise to paraffins and olefines which are of value directly or indirectly as detergent intermediates.

The *Fischer-Tropsch* process is the main synthetic source of higher aliphatic hydrocarbons, ignoring for the moment the polymerisation of olefines from other sources. Briefly, it consists of the reaction of carbon monoxide with hydrogen over a suitable catalyst such as cobalt or iron and gives rise to a complex mixture of products containing mainly paraffins and olefines together with some primary alcohols and other oxygenated compounds. The composition depends on the ratio of the reactants and on the conditions of reaction: a comprehensive account of the subject is given by Storch, Golumbic, and Anderson.[96] Interest has also been found in modifications of the *Fischer-Tropsch* process to provide a source of alcohols. The *I.G.* in particular were developing the so-called *Synol* process in which a reduced magnetic iron oxide promoted with alumina and potassium oxide is used as catalyst. This gives rise to improved yields of alcohols which are essentially straight chain. The yield of alcohols in the detergent chain length, however, is only of the order of 10 per cent of the total liquid products and clearly the *Fischer-Tropsch* process or its modifications cannot be regarded as a direct source of detergent alcohols unless as by-products of the industry. *Ruhrchemie* were interested in a similar process in which the iron catalyst is activated with vanadium or thorium: their procedure was believed to give mainly branched chain primary alcohols.[5] The *Fischer-Tropsch* process has been intensively studied in the U.S.A. and plant has been erected in recent times but though suitable alcohols are present these apparently have not yet been exploited commercially.

Processes to produce detergents from paraffins naturally differ widely from those applicable to olefines and though many of the end products are of very similar constitution it has been decided to deal with these separately for the sake of simplicity. Further, the use of linkages to link hydrophobic groups to the solubilizing group is not so prominent as it is with products derived from the fatty esters though the same methods are frequently employed. The subject-matter has therefore been sub-divided into the following categories:

1. Products derived from paraffins (sulphates, sulphonates, and carboxylic acid salts).

(*a*) Alkane sulphonates.

(*i*) Alkane sulphonic acid derivatives.

(*b*) Alkylaryl sulphonates.

(*c*) Miscellaneous products derived from paraffins.

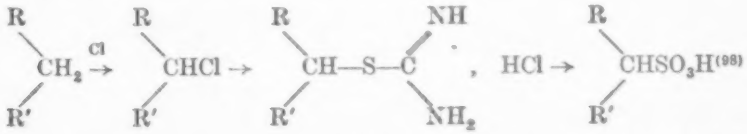
2. Products derived from olefines (sulphates, sulphonates and carboxylic acid salts).

(*a*) Alkane sulphonates.
(*b*) Sulphated primary alcohols.
(*c*) Sulphated secondary alcohols.
(*d*) Alkylaryl sulphonates.
(*e*) Substituted alkylaryl derivatives.
(*f*) Miscellaneous products derived from olefines.

1. *Products derived from paraffins (sulphates, sulphonates and carboxylic acid salts).*

Paraffins on controlled oxidation can give rise to mixtures containing secondary alcohols and ketones: the ketones can then be reduced back to alcohols. Yields, however, are poor. The further oxidation to carboxylic acids, however, has been developed commercially in Germany using *Fischer-Tropsch gatsch* (wax fraction) and oxidizing with air in the presence of a permanganate catalyst.[97] Fatty acids of the desired C_{12}—C_{18} range and essentially straight chain, can be obtained by fractional distillation of the crude acids and these can be hydrogenated to alcohols for sulphation or used for making the other derivatives already described. The higher paraffins can also be cracked to give olefines for use as detergent intermediates but this will be dealt with later. The principal methods are by conversion to alkane sulphonates and alkylaryls for sulphonation.

(*a*) *Alkane sulphonates.* The n-alkane-1-sulphonates in the C_{12}—C_{18} detergent range have been made by academic methods and studied, but there is as yet no practicable method which would make them readily available. In any event, the low solubility of their calcium and magnesium salts would make them of little technical value. Paraffins such as kerosene can be chlorinated and converted via the isothiuronium salts to sulphonic acids:



(98)

By far the most important route to the alkane sulphonates is the *Reed* reaction[99] which has been studied intensively by the *duPont* company in

America and the *I.G.* in Germany. It provided the major source of synthetic detergents in Germany during the war. The basic reaction involves treating a saturated hydrocarbon residue with chlorine and sulphur dioxide at about 30°C under the influence of actinic light (or other free radical catalyst) whereby the sulphon chloride is formed: this is subsequently hydrolysed:

$$R{-}CH_2 - R' + SO_2 + Cl_2 \rightarrow R{-}CH(SO_2Cl){-}R' + HCl$$
$$\rightarrow R{-}CH(SO_3Na){-}R'$$

The reaction can be applied to substituted hydrocarbons but for detergent purposes paraffinic hydrocarbons such as a highly refined Pennsylvania white oil are used. The *I.G.* employed a C_{12}—C_{18} fraction B.p. 220 to 320°C from *Fischer-Tropsch* oils known as *Kogasin* which was hydrogenated to convert the olefines and oxygen compounds present to a saturated hydrocarbon mixture, *Mepasin*. The sulphon chlorides were known in Germany as *Mersols* and the sodium sulphonates as *Mersolats*. An account of the *I.G.* process is given by VLUGTER.[100]

The reaction takes place by a free radical mechanism and ASINGER[101] has shown that substitution occurs in a random manner along the chain so that a mixture of sulphonates is eventually produced. Some chlorination and formation of disulphon chlorides occur, increasing in amount as the degree of conversion of oil to monosulphon chlorides is increased. In practice, therefore, a slight excess of sulphur dioxide over chlorine is used and as the disulphonated products are inferior in properties to the monosulphonates the best products are obtained if the extent of sulphochlorination of the oil is limited to about 30 per cent as in the case of *Mersol* 30. This of course introduces complications in that it is necessary to recover and re-use unreacted oil. Many means of doing this have been described, e.g. extracting the sulphon chlorides with liquid SO_2 or separating the oil mechanically after hydrolysis and dilution.

Sulphuryl chloride can be used[102] in place of sulphur dioxide and chlorine, the reaction being catalysed by light or other catalysts for free radical reactions such as peroxides.

An interesting alternative to the *Reed* process, the *Hostapon* process, was under active development by the *I.G.*[103] This involves reaction of the paraffin with sulphur dioxide and oxygen whereby the sulphonic acids are produced in one stage thereby effecting an economy in the use of caustic soda as compared with the *Reed* process. The reaction is catalysed by light but a preferred procedure is to react in the presence of acetic anhydride, initiating the reaction with hydrogen peroxide: presumably acetyl peroxide is formed thereby. The mechanism of the reaction has been studied and shown to proceed in two distinct stages, the second stage being best effected in the presence of water:

$$RH + SO_2 + O_2 + (CH_3CO)_2O \rightarrow RSO_2O_2COCH_3 + CH_3CO_2H$$

$$RSO_2O_2COCH_3 + 6RH + 6SO_2 + \frac{5}{2}O_2 + H_2O \rightarrow 7RSO_3H + CH_3CO_2H$$

The second equation summarises the total production of sulphonic acids in a reaction proceeding by a chain mechanism. The optimum conditions for the two stages are different and a continuous process in which they were effected in separate vessels was elaborated. The overall procedure is a somewhat complicated one, involving as it does a recovery of acetic acid as well as of unreacted paraffin. Free radical catalysts of the type of azodii*iso*butyronitrile,

$$Me_2C(CN)—N=N—C(CN)Me_2$$

can also be used for this reaction.[104] As a further alternative hypochlorous acid or chlorine monoxide together with sulphur dioxide can be used.[106] Thionyl chloride plus chlorine can be used to give sulphinyl chlorides and the sulphinates oxidised to sulphonates.[107]

The *Mersolats* are regarded as inferior to other types in detergent efficiency and they can only be compounded in limited amounts into powder formulations on account of their sticky and hygroscopic character. The bulk of German experience, however, was with *Mersol D* and *Mersol H* in which about 80 per cent and 50 per cent respectively of the oil was converted to sulphon chlorides rather than with the better products obtainable with a lower degree of sulphochlorination. It seems probable that the extensive use of the *Mersolats* was due largely to war-time conditions as it has not so far been paralleled elsewhere. Nevertheless, patenting of process improvements still continues in Germany;[108] the references given to alternative processes are also of recent date.

(i) ALKANE SULPHONIC ACID DERIVATIVES

Just as the fatty acids have been converted to a host of derivatives, so have the alkane sulphonyl chlorides from the *Reed* process been studied as intermediates for detergents giving products where the —CO— group of the former is replaced by the —SO_2— group of the latter, e.g.:

$RSO_2Cl \rightarrow RSO_2NH$—⬡ $\rightarrow RSO_2NH$—⬡—SO_3Na[13]

$RSO_2Cl \rightarrow RSO_2N(Me)C_2H_4SO_3Na$[109]

$RSO_2Cl \rightarrow RSO_2NHC_2H_4OH \rightarrow RSO_2NHC_2H_4OSO_3Na$[13]

$RSO_2Cl \rightarrow RSO_2NH_2 \rightarrow RSO_2NHCH_2CN \rightarrow RSO_2NHCH_2CO_2H$[13]

$RSO_2Cl \rightarrow RSO_2NH_2 \rightarrow RSO_2NH(C_2H_4O)_nH \rightarrow RSO_2NH(C_2H_4O)_nSO_3Na$[13]

$RSO_2Cl \rightarrow RSO_2O$—⬡ $\rightarrow RSO_2O$—⬡—SO_3Na[110]

These products apparently do not possess detergency characteristics sufficiently outstanding to merit the extra cost of manufacture.

(*b*) *Alkylaryl sulphonates.* Commercially, this is the most important class of synthetic detergents. Two technical routes are available, one from paraffins, which is considered here, and one from olefines which will be dealt with later.

The route from paraffins involves chlorination, condensation of the alkyl chlorides with benzene, followed by sulphonation:

The paraffins used may be based on natural petroleum or *Fischer-Tropsch* synthesis products, the former being developed mainly in the U.S.A. by *Monsanto and Allied Chemical & Dye Corporation* and the latter in Germany by the *I.G.*

Starting from petroleum it is usual to use a refined paraffinic fraction B.p. 220 to 240°C, consisting mainly of C_{12}—C_{13} paraffins. The chlorination is a random reaction giving rise to substitution in all the possible positions on the hydrocarbon chain[111] and poly-substitution can also occur, thereby giving rise to products of inferior quality. It is customary to limit the degree of chlorination to not more than 50 per cent of that theoretically necessary to convert to monochloroparaffin. The reaction can be effected at 60 to 90°C in the absence of a catalyst though catalysts such as light and iodine may be used. The crude alkyl chloride, frequently referred to as keryl chloride to denote its origin from kerosene, is then condensed with benzene using aluminium chloride in the normal *Friedel Crafts* technique at temperatures of 10 to 40°C and the crude, after destroying the catalyst, is distilled to recover unreacted benzene, paraffins and the alkylbenzenes.

The alkylbenzene can be sulphonated under a variety of conditions ranging from the use of 98 per cent sulphuric through oleum to sulphur trioxide at temperatures below zero to about 50°C but as the market requires a high quality product with respect to colour, odour and active agent content, it is normal to use mild sulphonating conditions consistent with substantially complete sulphonation. For example, the alkylaromatic may be sulphonated with 20 per cent oleum at 30°C and the spent acid removed by diluting to 80 per cent strength and separating at about 55°C. Useful surveys of conditions of sulphonation have been provided by PAULSON and by KIRSCHER.[112] Continuous processes have been elaborated. The most recent trend is apparently towards the use of sulphur trioxide (or oleum)[113] in conjunction with inert diluents such as air, propane or liquid sulphur dioxide, and intimations in the American press suggest that such a process is a commercial operation there. The merits of sulphur trioxide are of course obvious in the avoidance of a waste acid problem and freedom from inorganic salts.

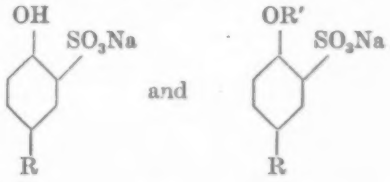
The *I.G.* route was based principally on materials from the *Fischer-Tropsch* process. Two different techniques appear to have been employed, one in which a *Kogasin* fraction B.p. 220 to 280°C, iodine value 11, was used directly,[12] and one in which the hydrogenated *Kogasin*, *Mepasin*, B.p. 230 to 320°, was first split into three fractions by distillation.[9] In the former, the crude alkylbenzene was sulphonated after removing benzene and paraffins, and in the latter the alkylbenzenes were distilled and the fractions combined before sulphonation. Doubtless the latter procedure would give the better product but

would be more expensive. A recent patent[114] emphasises the desirability of purifying at the various stages.

While aromatic intermediates other than benzene can be used for these alkylaryl sulphonates, benzene is preferred though there is no clear statement as to whether this is on grounds of costs or properties. The orientation of the sulphonic acid group in relation to the long chain alkyl group will, of course, depend on the original aromatic hydrocarbon used but no information is available on the effects which this may produce.

The alkylaryl sulphonates under this classification are stable to acids and alkalis, have good foaming powers and find extensive outlets in industrial and domestic use, the latter particularly when compounded into powder form for heavy duty detergency.

(*c*) *Miscellaneous products derived from paraffins.* Instead of condensing the chlorinated paraffins with aromatic hydrocarbons as just described, phenols and phenol ethers may be used giving rise to products of the types



Products of this nature are reported[115] as fully effective detergents as their benzene analogues but they would be more expensive to produce. Condensation has also been effected with phenylacetic acid to give the alkylphenylacetic acids. Alternatively, the alkylbenzene can be reacted with acetyl chloride and the keto group oxidised to the carboxy acid.

The chloroparaffins may be dehydrochlorinated to give another source of olefines or converted to other useful intermediates, e.g. by reaction with amines and glycols, though reactions of this nature involving alkaline conditions usually result in relatively poor yields of the desired derivative. Nitration of the higher paraffins and reduction of the products to amines has also been studied but so far without commercial success.[5]

A recently discovered route[105] to alkyl sulphuric esters which involves oxidising paraffins to hydroperoxides followed by reaction with sulphur dioxide:

$$RH \rightarrow R{-}OOH \rightarrow R{-}O{-}SO_2{-}OH$$

seems of potential interest. It is reminiscent of the *Hostapon* process (p. 118) though conditions and end products are different.

2. *Products derived from olefines.*

Olefines of wide variety are available from the petroleum industry and from synthetic sources such as the *Fischer-Tropsch* process. Briefly, they may be separated into two classes for use as detergent intermediates, namely the longer chain olefines of C_{10}—C_{18} obtained by cracking or distillation processes

or from the *Fischer-Tropsch* process and those built up by polymerisation of lower olefines such as propylene and isobutylene: both are of great technical importance.

(*a*) *Alkane sulphonates.* Although several methods are available for making sulphonic acids from olefines, none has achieved technical importance. Olefines such as tri*iso*butylene can add on hydrogen sulphide and the mercaptan oxidised. Alternatively, the olefine may be reacted with sulphur and the sulphide oxidised or it may be reacted with sulphur dichloride and the mustard gas analogue reacted with sodium sulphite. Vigorous sulphonating conditions can give rise to hydroxysulphonic acids:

$$RCH = CHR' \rightarrow RCH(OSO_3H)CH(R')SO_3H \rightarrow RCH(OH)CH(R')SO_3Na$$

This latter technique was employed by the *I.G.* in sulphonating octadecene to obtain an emulsifying agent.[12] In the case of olefines containing the grouping $—CH=C(CH_3)CH_3$ sulphonation with sulphur trioxide-dioxan complex gives sulphonic acids of the type $—CH=C(CH_3)CH_2SO_3H$.[116]

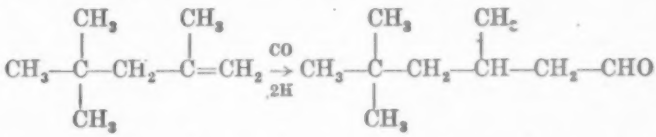
The addition of sodium bisulphite to unactivated double bonds does not proceed easily but recent work[117] with improved catalysts such as inorganic oxidising agents and peroxides with a tertiary alkyl group attached directly to the oxygen, together with more rigorously controlled reaction conditions, has shown greater promise.

(*b*) *Sulphated primary alcohols.* The acute shortage of natural fats and oils in Germany during the last World War drove the Germans to develop other sources of alcohols for detergent purposes. Production via fatty acids from *Fischer-Tropsch gatsch* has already been mentioned. Of greater interest however is the so-called *Oxo* process. This reaction, first reported by Smith *et al.*[118] and developed primarily by Roelen and co-workers of *Ruhrchemie A.G.*, consists in reacting an olefine with carbon monoxide and hydrogen to give aldehydes which are reduced to alcohols, thus:

$$\begin{array}{l} \qquad\qquad\qquad\qquad RCH_2—CH(R')CHO \xrightarrow{2H} RCH_2—CH(R')CH_2OH \\ RCH = CHR' + CO + 2H \\ \qquad\qquad\qquad\qquad RCH(CHO)—CH_2R' \xrightarrow{2H} RCH(CH_2OH)—CH_2R' \end{array}$$

where R′ may be hydrogen or an alkyl residue. A description of the process as operated by the *I.G.* is given by Holroyd *et al.*[119] and a full account of the *Oxo Gesellschaft's* process by Holm *et al.*[120] (cf. also ref. (96)). The process is applicable to any olefinic material of suitable chain length for detergency ($C_{11}—C_{18}$) but was designed principally for *Fischer-Tropsch* gas oil fractions. Basically, the olefinic fraction containing about 3 per cent of the normal *Fischer-Tropsch* cobalt catalyst is reacted with carbon monoxide and hydrogen at 125 to 145°C and 200 atm until no further absorption takes place—about 20 to 30 minutes. The pressure is released to allow the escape of carbon

monoxide which interferes with the hydrogenation step and the product is then reduced with hydrogen under substantially the same conditions as in the earlier stage. Yield of alcohols is about 80 per cent on olefines, by-products arising from the polymerisation of olefine and aldolisation of the aldehyde. The conditions of the carbonylation stage are varied somewhat according to the particular olefine to be reacted. The reaction, according to the above equations, gives a mixture of isomeric primary alcohols and in the case of a Δ^{-1} olefine this would be a mixture of the normal alcohol and the α-methyl isomer. In actual fact, however, the cobalt catalyst causes some migration of the double bond so that related branched chain primary alcohols of the same molecular weight but differing in extent of branching are also produced. The actual composition of the product will therefore be affected not only by the starting material used but also by the conditions of preparation. According to HOLM *et al.* (*loc. cit.*) mainly α-methyl and α-ethyl branching occur with Δ^{-1} olefines under the conditions described by them. In the case of branched chain olefines addition of the aldehyde group takes place preferentially at the position giving rise to the least steric effects so that with di*iso*butylene, for example, substantially one individual product is produced:



The *Oxo* reaction has of course been extensively examined as a source of alcohols in general. For detergents, olefines of suitable chain length from the direct normal *Fischer-Tropsch* process, from modified *Fischer-Tropsch* processes, from the cracking of higher paraffins and from the polymerisation of lower olefines, etc., have been examined. Propylene polymers (C_9—C_{15}) and tri- and tetra-*iso*butylene gave poorer conversion to alcohols. Properties will be discussed later.

The use of material such as *Fischer-Tropsch* gas oil as starting material for the *Oxo* process brings its own difficulties in that, being a mixture of olefines and paraffins of differing chain length and the olefines also varying in the position of the double bond, the resultant alcohol-paraffin mixture cannot be separated into its desired constituents by distillation. It was proposed, therefore, to fractionate the oil into C_{11}—C_{12}, C_{13}—C_{14}, etc. fractions, to treat these separately, to isolate e.g. the C_{12}—C_{13} alcohols from the C_{11}—C_{12} paraffins by distillation and then to combine all the alcohols for sulphation. Other methods, such as treating with boric acid to convert the alcohols to boric esters and then separating, were also considered.

Sulphated *Oxo* alcohols have been extensively studied in Germany and their properties are summarised by BRANDNER *et al.*[13] They were to have been the answer to the shortage of natural fats. Branched chain alcohol sulphates are more soluble than straight chain products containing an equal number of carbon atoms: detergent properties are the better the nearer the alcohol group

is to the end of the chain. α-Methylstearyl alcohol is reported to give a product as soluble as, and practically as good a detergent as, oleyl alcohol. For these reasons, it was preferred to use alcohols derived from Δ^{-1} olefines such as were produced to a larger extent by wax-cracking than in the normal *Fischer-Tropsch* synthesis. Some workers were of the opinion that the *Oxo* process was more valuable for the C_{10} to say the C_{14} olefines than for the higher olefines inasmuch as the additional carbon atom introduced brought them into the better chain length for detergency. In general, it would appear that *Oxo* alcohol sulphates can be produced equal in detergency to the straight chain alcohol sulphates but the branched chain structure results in softer products which might cause difficulty in bulk production of spray-dried powders.

REPPE[121] has discovered a new reaction analogous to the *Oxo* reaction involving reaction of an olefine with a metal carbonyl and water to give carboxylic acids:

$$R—CH=CH_2 + Ni(CO)_4 + H_2O \rightarrow RCH(CH_3)—CO_2H + RCH_2CH_2CO_2H$$

These acids can then be reduced to alcohols. This procedure has been stated to give better alkyl sulphates than the *Oxo* process though it is not clear why there should be any difference unless perhaps there are differences in the amounts of the various isomers formed.

(c) *Sulphated secondary alcohols.* Direct sulphation of olefines is by far the most important route to the secondary alcohol sulphates and has been intensively developed in Europe by the *Shell* organization and the *Anglo-Iranian Oil Company.* It was also under consideration by the Germans but does not appear to have found favour in the U.S.A. though it has also been studied there. Earlier work on this type was based on olefines obtained, for example by dehydration of alcohols, by the distillation of spermacetti giving cetene, or by chlorination of paraffins followed by dehydrochlorination. Technical progress, however, awaited development of cheap sources of olefines by the petroleum or related industries.

The sodium salts of the secondary alkyl sulphates derived from straight chain olefines in the C_{12}—C_{18} range with the double bond at or near the α-position are the simplest and possibly the cheapest detergents based on petroleum-type products. The cracking of paraffin wax or *Fischer-Tropsch gatsch* provides one source, the distillation of oil-bearing shale a second; the *Synol* process giving a mixture containing both alcohols and olefines, as well as the normal *Fischer-Tropsch* process provide alternative routes. Branched chain olefines in the C_{12}—C_{18} range from the polymerisation of lower olefines such as propylene and *iso*butylene do not sulphate satisfactorily. The routes from paraffin wax and shale oil have been described by BIRCH.[122] Wax cracking can give rise to a feed-stock containing 60 to 70 per cent of olefines whereas the olefine content of the shale oil fractions is much lower at 25 to 30 per cent. According to BIRCH, the olefines are sulphated at 10 to 15°C using a molar ratio of acid to olefine of about 2 : 1, the critical operating conditions being efficient mixing and short contact time. The concentration of acid employed

ranges from 90 to 96 per cent depending on the feed-stock and the precise conditions of sulphation. A preferred shale oil feed-stock has B.p. 200 to 300°C and is sulphated with 96 to 98 per cent acid at 20°C.[123]

Difficulties arise in the isolation of the sulphated products due to the fact that not only are crude olefines used but also the sulphation gives rise to dialkyl sulphates:

$$2R—CH = CH_2 + H_2SO_4 \rightarrow R—CH(CH_3)—O—SO_2—O—CH(CH_3)R$$

and olefine polymerisation products. It is customary to neutralise and hydrolyse the dialkyl sulphates with caustic. The latter step would be expected to give the sodium alkyl sulphate and alcohol, but according to BIRCH the reaction results also in the formation of olefine. When hydrolysis is complete the product is cooled and alcohol added to facilitate the removal of extraneous products which is effected with a suitable hydrocarbon solvent. In the case of shale oil olefines, acid sludge is removed by centrifuge before hydrolysis and purification.

The *I.G.* developed a special technique for the continuous sulphation of olefines, the main feature of which lies in a reactor of novel design.[13] This comprises a jacketed cylinder inside of which revolves another cylinder, the annular clearance being 1 mm on the pilot plant scale where the external cylinder was 50 cm long by 8 cm in diameter. The inner cylinder supported from the top is rotated at 120 r.p.m. The acid is fed in from the side and the olefine to the centre of the bottom of the outer cylinder. Cooling is effected with methyl chloride or brine in both the jacket of the outer cylinder and the inside of the inner cylinder.

The raw materials of interest to the *I.G.* were C_{14}—C_{18} olefines from cracked *Fischer-Tropsch* wax and suitable fractions from the *Synol* process. Sulphation is effected with a 15 per cent excess of acid to minimise as far as possible the formation of dialkyl sulphates. The feed is adjusted to give a contact time of less than 30 sec. The temperature is kept below 10° depending on the freezing point of the olefine mixture: as low as — 20° had been used. The strength of the acid is varied according to the quality of the olefines, 98 per cent acid being suitable for very pure olefines while fuming sulphuric acid of varied strength is used for less pure olefines and for the *Synol* olefine-alcohol mixtures. The sulphation mass is neutralised continuously with caustic and the working up procedure is substantially that described above. Under the conditions used it was estimated that about 20 per cent of the olefines were converted to dialkyl sulphates.

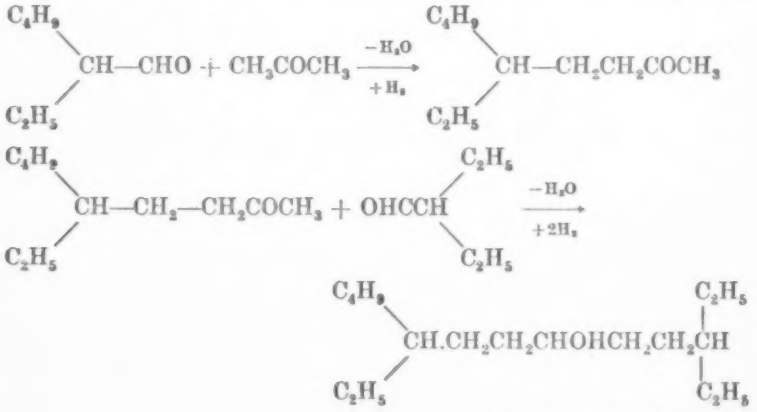
A comparison of the procedure described by BIRCH with that of the *I.G.* is interesting in that though they show many features in common the necessity of practical study to obtain optimum conditions in particular cases is clearly revealed, for example in the strength and quantity of acid used.

The more recent patents[124] in this field are concerned principally with improved techniques, products or economies. Thus, sulphation is done by feeding the reactants tangentially into a mixing nozzle which sprays the mixture in a finely divided form tangentially into a cooled annular chamber; a

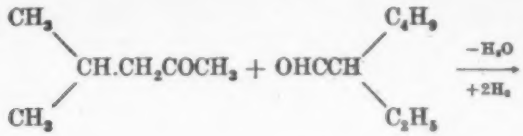
shale oil feed-stock is subjected to a solvent refining process to obtain better colour and odour and by-products are utilised in various ways. An interesting account of the effect of conditions of sulphation using pure Δ^{-1} olefines has been given by KOOLJNAN.[125]

From the interesting work of DREGER *et al.*[126] who have studied a series of pure secondary alcohol sulphates it is seen that detergency, as is the case with the *Oxo* alcohol sulphates, is the better the nearer the sulphate group is to the end of the chain and the greater the chain length within the range studied. Properties of the technically derived products will, of course, depend on the olefine feed-stock used and the purity achieved. The *I.G.* apparently preferred a C_{14}—C_{18} cut but economics will demand the use of as wide a fraction as is possible consistent with acceptable properties. The technical products have good detergent properties though they are better for wool than for cotton: their low cost is an attractive feature. Evaluation of a product from an olefine cut of average mol wt 221 (C_{15}—C_{16}) by the *I.G.* showed it to be intermediate in properties between sodium lauryl sulphate and sodium oleyl sulphate. Difficulties have been experienced in producing sulphated olefine products in the form of free-flowing powders though this property too will depend to some extent on the olefines used: it can be overcome by grinding with non-caking diluents. There was some difference of opinion in Germany as to the relative merits of the *Oxo* alcohol process and direct sulphation of the olefines as routes to detergents; the *I.G.* leaning was towards the latter whereas *Ruhrchemie* and *Henkel* thought that the better properties given by the *Oxo* alcohol sulphates justified the cost of the extra operations involved. The secondary alcohol sulphates are stable under alkaline conditions but are hydrolysed rather readily by acids.

The lower aldehydes and ketones, which for convenience can be regarded as based on olefines from the natural or synthetic petroleum industry, can be used to synthesise higher secondary alcohols; these have a highly branched structure. The usual aldol type condensation followed by reduction is employed, e.g.:



3 : 9-diethyltridecan-6-ol

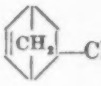


2-Methyl-7-ethylundecan-4-ol

Sulphated derivatives of such alcohols have been marketed in the U.S.A. under the names *Tergitols* 4 and 7 but their technical interest is primarily on account of their good wetting properties. Sulphation of secondary (and tertiary) alcohols is more difficult to effect satisfactorily than the sulphation of primary alcohols owing to their tendency to form olefines by dehydration and modified sulphation conditions are preferred.[128]

(*d*) *Alkylaryl sulphonates.* Alkylaryl sulphonates derived from chlorinated alkanes have already been described. Such a route fits well into the organic chemical industry but the alternative route in which the alkyl group is derived from an olefine is more suited to the petroleum industry and, although apparently first used in Germany by the *I.G.*, it has been extensively developed in the U.S.A., especially by *Standard Oil*, and is now of major importance as a source of synthetic detergents.

While a wide variety of olefines can be obtained and used, e.g. those from the cracking of paraffin wax or *Fischer-Tropsch* type processes, technical interest, presumably on grounds of ready availability and economics, has centred on polymers of C_{12}—C_{16} chain length from the lower olefines. Polymers containing tertiary carbon atoms derived for example from *iso*butylene are, however, not suitable for the alkylation of the simple aromatic hydrocarbons such as benzene as depolymerisation occurs under these conditions giving rise to lower molecular weight alkylbenzenes which are unsuitable as detergent intermediates. A correlation between the tendency for polymer disproportionation and infra-red spectra has been noted.[129] Propylene polymers, and particularly the tetramer, are the preferred olefines. The propylene, which should be as free as possible from butylenes, is polymerised using e.g. a phosphoric acid catalyst in a silver-lined reactor at 180 to 200°C and 40 to 60 atm and the required polymer fraction separated by distillation.[12] It is best to use as close a fraction as possible. The precise structure of the tetramer has not yet been described, but clearly it will be a highly branched alkene and presumably dependent on conditions of polymerisation. Polymers of the structure $RCH{=}CHR'$ rather than $RC(R'){=}CH_2$ have been preferred and conditions for producing such, described.[130] Other processes for preferred olefine polymers and procedures for cleaning up the olefines before use, etc., are described.[131]

Olefine-cyclopentadiene adducts of the type  can be used as alkylating agents.[132]

Of the aromatic hydrocarbons used, benzene is the most generally accepted, though toluene and naphthalene—the latter with a C_9 alkyl residue—are also employed. Various products of this type are available and advice on sulphonation can frequently be obtained from the suppliers.

Catalysts employed for the alkylation are of the type normally used in such reactions. Hydrofluoric acid seems to be preferred for alkylating benzene which is less reactive than toluene or naphthalene though aluminium chloride or sulphuric acid can be employed and are apparently quite satisfactory in the case of toluene or naphthalene. After separation of condensing agent, especially easily effected in the case of hydrofluoric acid which is recovered for re-use, the alkylate is distilled to remove unreacted materials and any lower alkylbenzenes formed, and the desired fraction collected. Where hydrofluoric acid is employed traces of combined fluorine are eliminated, e.g. by heating with bauxite at 100°C. Sulphonation is carried out by the methods already described but as there is some tendency for de-alkylation to occur conditions must be chosen with care and the malodorous by-products extracted with a suitable solvent if need be.

Details of the *I.G.* process have been given.[133] They, however, were obliged to use a rather wide polymer fraction and detergent properties were therefore not as good as can be obtained: products from benzene and toluene are stated to be similar to one another in detergency.

The alkylbenzene sulphonates from olefines are similar in general properties to those from the chlorinated alkanes and find extensive outlets as general purpose synthetic detergents. Properties will again be dependent on the olefines used as well as on processing conditions. Products of the type

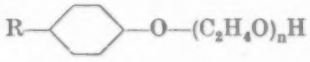
where R is straight chain (derived from Δ^{-1} olefines) of 8 to 11 carbon atoms are stated to have outstanding detergency[134] but obviously the theoretical structural variations possible in alkylaromatics as a whole are enormous and the approach has been technological rather than academic.

(*e*) *Substituted alkylaryl derivatives.* Olefines of the types already described can be reacted readily with some substituted aromatic hydrocarbons and especially so with phenols and phenol ethers. With phenols the ease of alkylation permits the use of tertiary carbon olefines such as di*iso*butylene which cannot be used with benzene so that a still greater range of detergent intermediates can be obtained. These have been widely examined but their main use has been for making non-ionic types.

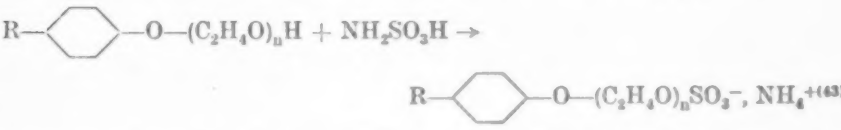
A phenol of the type

where R is at least 8 carbon atoms may be taken as a general example. Such phenols may be sulphonated directly giving effective detergents if R is at least 12 to 14 carbon atoms.

The alkylphenols can be reacted with chloracetic acid to give the alkylphenoxyacetic acids which have some detergent properties in themselves or the phenoxyacetic acids may be used as intermediates in the wide variety of ways which have been described for the fatty acids giving e.g. analogues of *Igepon A*, *Igepon T* and *Medialan A*. They can be etherified with alkyl groups (or the phenol ethers may be alkylated) and sulphonated. One procedure which has proved of commercial value is to make the polyethenoxy ether

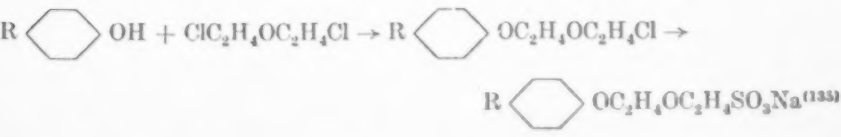


by reaction with ethylene oxide and sulphate the terminal hydroxyl group. This can be done by the normal procedures used for sulphating fatty alcohols though the *I.G.* used sulphamic acid available to them as a by-product:

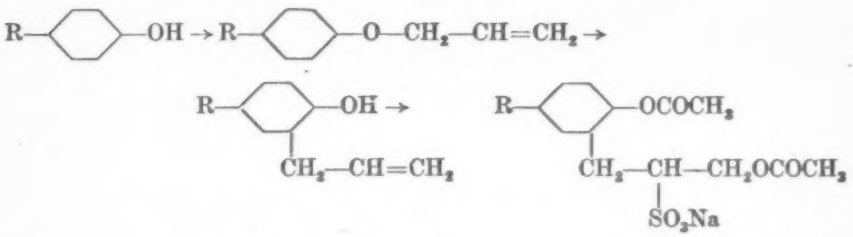


the ammonium salt being subsequently converted to sodium salt. According to the *I.G.*, such products have the merits over the straight polyethenoxy products (discussed later) of economising in the more expensive ethylene oxide, one sulphate group being roughly equivalent in solubilising power to four ethenoxy groups, of producing better foaming power and of not having the negative temperature-solubility coefficient characteristic of the polyethenoxy detergents. They have, however, the demerits of acid instability and substantivity to protein fibres. The preferred *I.G.* detergent for cotton and general household purposes was of this type where the phenol residue carries two C_6—C_7 groups and n is 4 (*Alipal D*). Products where $R = C_{12}$ and $n = 3$ (*Alipal C1*) and from a dodecylcresol where $n = 6$ (*Igepal B*) were also produced. These sulphated products cannot easily be spray dried to powder forms and are marketed as aqueous solutions. Similar products have appeared in the U.S. market, e.g. *Triton W* 30.

Sulphonic acid groups can be introduced into the molecule indirectly, e.g.:

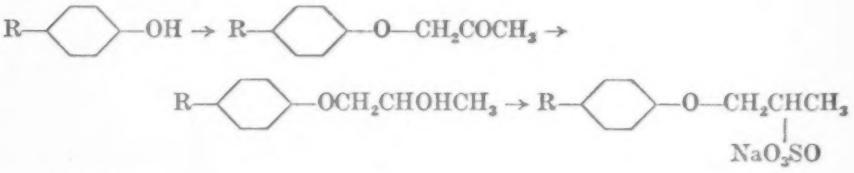


and some commercial products, e.g. *Triton* 720, belong to this class. The *I.G.* were interested in a rather more complicated procedure which involves making the allyl ether, rearranging to the C-allyl phenol and sulphonating in the presence of acetic anhydride:



High temperatures during neutralising or drying cause hydrolysis of the acetyl groups though the effect of this was not known.[13]

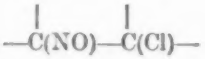
The intermediate allyl ethers can be sulphated and products of the same structure obtained by condensing the alkylphenol with chloroacetone, reducing the keto group to hydroxyl and sulphating:[136]



The alkylphenols can also be reduced to alkylcylohexanols giving further alternatives to the fatty alcohols as detergent intermediates for sulphation etc. Though potentially quite attractive economically this route does not appear to have been exploited commercially. The Kolbe reaction gives alkylsalicylic acids which are surface active.

Sulphonic acid groups can be introduced into the side chains e.g. by reacting the alkylaromatic with an alkene sulphonic acid and by means of the *Reed* reaction. Two alkylaryl groups may be linked together by means of an aldehydosulphonic acid. Alkylaryl sulphonic acids have been converted to their sulphon chlorides and reacted with e.g. sulphanilic acid giving

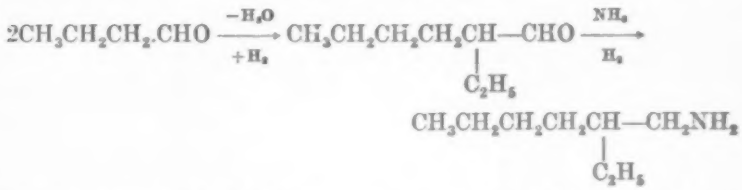
(*f*) *Miscellaneous products derived from olefines.* An unusual type of detergent which may be compared in structure with the alkane sulphonates can be obtained by reacting an olefine with nitrosyl chloride followed by a mixture of sodium sulphite and bisulphite. The chloro group of the intermediate nitrosoalkyl chloride:



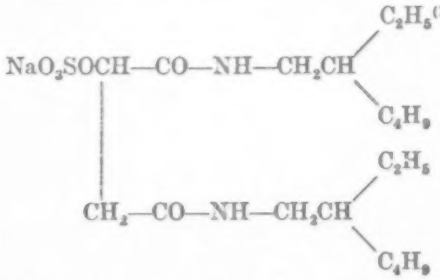
is converted to sulphonate but the nitroso group may give rise to keto, amino and sulphamic acid groups so that the final product is a complex mixture.[138] A product of this type has been marketed in the U.S.A. as *Nytron* as a general purpose detergent; it is recommended for cleaning surfaces contaminated with radioactive materials.

The olefines can also be converted to glycols for sulphation, to chlorhydrins for reaction with sodium sulphite, condensed with phenylacetic acid to give the alkylphenylacetic acid or with maleic anhydride to give alkenylsuccinic acids which in turn can be converted to soluble amide, etc., derivatives. The *Reed* reaction has been applied to olefines.[139] Mercaptans from the olefines can be reacted with diazonium salts[140] giving, e.g.:

Amines can be produced via the lower aldehydes and ketones, e.g.:

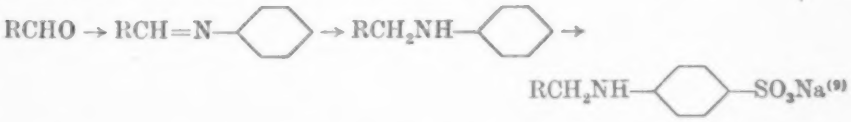


and these can be used in a manner analogous to the fatty amines giving e.g. the sulphate of the malic bis-amide:



[141]

Aldehydes of suitable chain length obtainable *via* the *Oxo* process are also of interest e.g. by reacting with an aromatic amine, reducing and sulphonating



[9]

C. Miscellaneous anionic solubilising groups

Although sulphonic acid and sulphuric ester groups are the principal means of solubilising the hydrophobic portion of the detergent molecule, with carboxylic acid groups coming a poor third, still other groups have been employed.

Intermediates carrying alcoholic and olefinic groups can be converted to persulphates by means of persulphuric acid:

$$ROH + H_2S_2O_8 \rightarrow R{-}OSO_2{-}O{-}O{-}SO_2Na^{(142)}$$

Such products are stated to possess bleaching as well as detergent properties.

Thiosulphates in considerable variety have been prepared by reacting halogeno compounds such as n-alkyl halides, chloracetyl and chlormethyl derivatives with thiosulphate:

$$ROCH_2Cl + Na_2S_2O_3 \rightarrow ROCH_2{-}S{-}O{-}SO_2Na^{(143)}$$

or sulphating mercaptans with sulphur trioxide complexes.[144] Hydrosulphite derivatives have been made by reacting the halogeno compounds with sodium hydrosulphite, $Na_2S_2O_4$.[145]

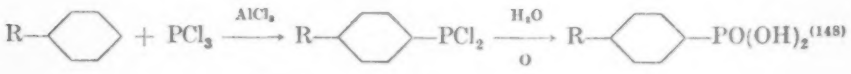
Long chain sulphonamides can be used but only under very alkaline conditions while the more acidic N-acyl and N-sulphonyl derivatives such as

$$RSO_2NHCOPh,\ PhSO_2NHCOR \text{ and } RSO_2NHSO_2R'^{(146)}$$

are soluble in sodium carbonate. The related sulphonyl ureas, $RSO_2NH\,CONH_2$, made by reaction of the sulphonamide with sodium cyanate are also soluble in weak alkalies and excellent detergency is reported to be given when R is cetyl.[147]

Considerable attention has been given to acid esters of the phosphorus acids which may be considered analogous to the sulphated alcohols, but these tend to be of indefinite constitution and of little practical interest because of the low solubility of the calcium salts.

Phosphonic acid derivatives in which there is a direct carbon-phosphorus bond and thus analogous to the sulphonic acids, can be obtained by such reactions as:



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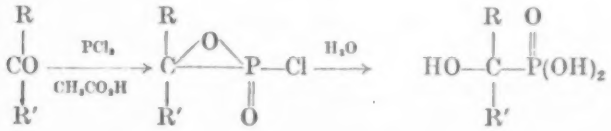


An interesting reaction akin to the *Reed* reaction has been described in which a paraffin is treated with phosphorus trichloride and oxygen to give the phosphonyl dichloride, $RPOCl_2$,[151] but the alkane phosphonates do not appear to have merited technical development.

III. Cationic Agents

Although of great technical importance as textile auxiliary products, bactericides, etc., the surface-active cationic agents are of little practical interest as detergents though detergent properties are claimed in many cases and they are sometimes known as "invert soaps." The reasons for this are probably associated with (*a*) relatively greater cost as compared with the common anionic and non-ionic types, (*b*) their high affinity for most solid surfaces, e.g. protein fibres and glass, which will reduce their efficiency and (*c*) their tendency to be oriented on such surfaces so that the hydrophobic portion of the molecule is turned towards the aqueous phase, thereby tending to reduce detergent efficiency and making the surfaces more difficult to wet. As an example, their use to give dispersions and emulsions substantive to wool clearly suggests that they would not be efficient detergents for wool. Only a brief outline of the cationic agents will therefore be given: greater details can be obtained from other sources.[2], [3]

Amine salts and quaternary ammonium salts are the main representatives of the cationic class and are treated separately: others are dealt with under a third heading.

A. Amine salts

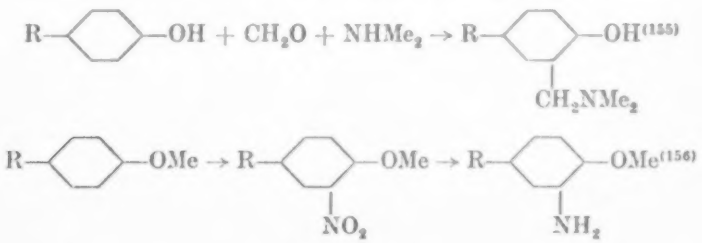
Salts of primary, secondary, and tertiary amines carrying a fatty chain can be used as scouring agents. The ingenuity shown in coupling the hydrophobic group to solubilising anionic groups has also been applied to means of attaching hydrophobic groups to basic groups, e.g. by means of ester and amide linkages such as:

$$R.CO_2H + Me_2NC_2H_4OH \rightarrow R.CO_2C_2H_4NMe_2$$ [152]

$$R.CO_2H + NH_2C_2H_4OH \rightarrow R.CO.NH.C_2H_4OH \xrightarrow{\text{acid}} R.CO_2C_2H_4NH_2 \text{ salt}$$ [153]

$$R.CO_2H + NH_2C_2H_4NMe_2 \rightarrow R.CO.NH.C_2H_4NMe_2$$ [154]

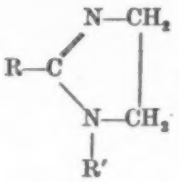
Likewise, aromatic and heterocyclic amine derivatives have been prepared and products based on both fatty acids and petroleum-type intermediates by the numerous devices available to the organic chemist, e.g.:



[155] [156]

In general, however, bases which have to be used as salts of acids could have only a very limited outlet as detergents apart from the disadvantages already

noted. It is of interest, nevertheless, to record that iminazoline derivatives of the type:



where R is a long fatty chain and R′ a short alkyl chain such as hydroxyethyl[157] are marketed in the U.S.A. (*Alro Amines* and *Amine* 220) and stated to have detergent properties.

B. Quaternary ammonium salts

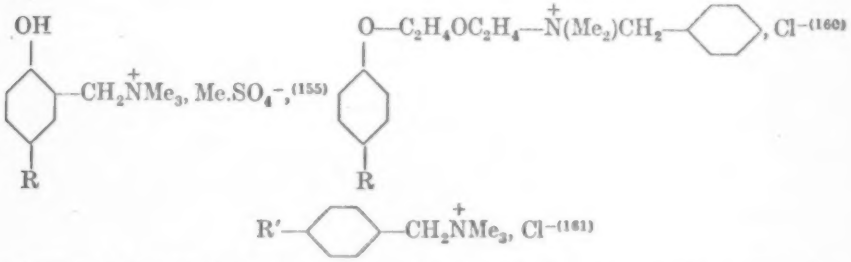
These constitute a large class of surface-active agents of technical importance but though they have some stability under alkaline conditions depending on the particular substance under consideration, the general factors already described make them of little practical interest for detergency as such.

The earlier work was based on fatty derivatives, e.g.:

$$R.OH \rightarrow R.Br \rightarrow R\overset{+}{N}Me_3, Br^-{}^{(158)}$$

$$R.CO_2H \rightarrow R.CO.NH.C_2H_4NEt_2 \rightarrow R.CO.NH.C_2H_4\overset{+}{N}(Me)Et_2,\ Me.SO_4^-{}^{(159)}$$

and has been extended in the same way as with the anionic types using a variety of linkages and other means of introducing quaternary ammonium groups. Petroleum-type intermediates have also been examined extensively, e.g.:



where R is the octyl group from di-*iso*butylene and R′ may be from an olefine such as propylene tetramer.

Quaternary ammonium hydroxides such as that from the reaction of ethylene oxide with dimethyloctadecylamine, namely dimethyloctadecyl-β-hydroxyethylammonium hydroxide, is stated to be an effective detergent:[162] it would, of course, be strongly alkaline.

C. Other cationic types

The search has also extended over the broad field of other solubilising basic groups such as amine oxides, imino ethers, amidines, guanidines, biguanides,

ternary sulphonium compounds and so on. Again, technical interest as detergents is negligible though such properties are frequently described in the patent literature. For example, the thetine chloride:

$$C_{12}H_{25}\overset{+}{S}(Me)CH_2CO_2H,\ Cl^-$$ [163]

from methyl dodecyl sulphide and chloroacetic acid is stated to be equivalent in detergent power to the sulphated fatty alcohols under acid, neutral and alkaline conditions. Under alkaline conditions the thetine is presumably formed: this could be regarded as akin to the betaines and so non-ionic.

IV. Non-ionic Detergents

Although the products which have been described all contain strongly hydrated ions, ionisation is not a necessary feature for detergency, the general requirement being a sufficient degree of hydration to solubilise the hydrophobic part of the detergent molecule. Non-ionising polar groups such as —O—, —OH, —CONH— and —COO— hydrate and if a sufficiency of these can be introduced, water-solubility will be obtained. From a practical point of view, the introduction of a plurality of ether groups by means of ethylene oxide[164] is by far the most easily effected and has achieved considerable industrial importance in Europe and America. It is perhaps of interest to relate that the initial development of the so-called polyethenoxy products arose from the fact that after the 1914–18 War the Germans were not allowed to make thiodiglycol which is an intermediate for mustard gas but which was also required as a printing assistant, and they therefore directed their attention to the oxygen analogue, diglycol, and hence to ethylene oxide reactions in general.

The non-ionic detergents are dealt with under the headings "polyethenoxy derivatives" and "miscellaneous non-ionic types."

A. Polyethenoxy derivatives

Ethylene oxide, $\overset{O}{\diagup\diagdown}$ CH_2—CH_2, is obtained from ethylene either *via* the chlorhydrin or by direct oxidation, both methods being employed in the U.S.A. It presents definite hazards in use and should be handled with due precautions. It is exceedingly reactive and in general it reacts with compounds containing a reactive hydrogen to give products containing the polyethenoxy grouping

$$—(C_2H_4O)_nH$$

where n can be varied at will. It should be borne in mind, however, that n is not usually a single integer. Thus, in reacting ethylene oxide with water in molar proportions, not only ethylene glycol but also diethylene glycol and higher polyethylene glycols are formed. Similarly with alcohols, polyglycol mono-ethers are produced in addition to glycol mono-ethers. In the case however of more acidic starting materials such as phenol the mono hydroxyethyl ether is reported to be the principal product formed with molar quantities of

reactants but further reaction with ethylene oxide results in a mixture of polyglycol phenyl ethers in which the proportions of the individual chemical entities follow a *Poisson* type distribution curve. Thus in the reaction of phenol with 3·43 mols ethylene oxide with caustic soda as catalyst, the following distribution where n is the number of ethenoxy groups has been reported by MILLER *et al.*[165]

n	=	1	2	3	4	> 4
Found	=	4·7%	17·8%	27·1%	27·1%	29·3%
calc.		4·9%	15·9%	24·0%	23·2%	32·0%

Similar results have been reported by MAYHEW and HYATT[166] who fractionated condensates of a nonylphenol with 6 and 9 mols ethylene oxide. These authors also show that there is little difference in detergency between the isolated fractions and unfractionated material of the same average ethenoxy chain length. Results reported[13] to have been obtained by the *I.G.* with a series of dodecanol derivatives, however, show that a higher ethenoxy chain length is required for maximum detergent efficiency with a pure polyglycol ether than with the total mixed condensate though there is little difference between these optimum values. In this connection it is of interest to note that more efficient emulsifying agents are obtained by using fractions of selected ethenoxy chain length from fatty alcohol condensates.

The distribution of the ethylene oxide over various chain lengths is explained on the grounds that the ion first formed, $R—O^-$, reacts with ethylene oxide to give the ethenoxy ion, $R—O—C_2H_4—O^-$. In the case of a phenol, proton transfer will obviously occur preferentially to give phenoxide ions so long as unreacted phenol remains but where alcoholic residues are involved the charge will be redistributed amongst them.

The versatility of ethylene oxide reactions and the fact that the molar ratio of ethylene oxide to raw materials can be varied at will can give rise to an infinity of products. In practice, however, the ratio is selected so as to balance the hydrophobic and hydrophilic characteristics according to the end use of the product. Reaction with ethylene oxide therefore offers advantages over other methods of solubilisation such as sulphation or sulphonation and can be used for example to obtain detergents from a C_8 alkylphenol where sulphonation results in too great an increase in hydrophilic properties. The range of raw materials which can be used for detergents is thereby greatly increased. For convenience the polyethenoxy products are sub-divided as follows:

1. Derivatives of acids.
2. Derivatives of alcohols.
3. Derivatives of amides.
4. Derivatives of amines.
5. Derivatives of mercaptans.
6. Derivatives of phenols.
7. Miscellaneous polyethenoxy products.
8. Properties of polyethenoxy products.

1. *Derivatives of fatty acids*

These are esters of the general formula

$$R.COO—(C_2H_4O—)_nH$$

Fatty acids from animal, vegetable and synthetic sources can be used depending on availability and economics. The polyethenoxy esters can be made by reacting a suitable polyethylene glycol with the fatty acid under normal esterification conditions: this process is stated to give some polyglycol diester, as might be expected. Polyglycols of various average molecular weights are available, particularly in the U.S.A., and this route can therefore be expected to appeal to the smaller manufacturer. Alternatively, the fatty acids can be reacted directly with ethylene oxide the normal method being to pass in the ethylene oxide at 2 to 3 atm pressure while maintaining the temperature at 150 to 180°C in the presence of an alkaline catalyst. Details of the *I.G.* procedures for their wide range of polyethenoxy products have been given.[12], [43] Nothing has been published on the chemistry of this reaction with fatty acids but work with acetic acid suggests that some di-esters might be formed:[167] further, conditions are such that ester interchange reactions could be expected to occur giving a mixture of various polyglycol esters. Products made by the two routes are stated[2] to be essentially similar in properties. About 10 to 15 mols ethylene oxide are required to bring the C_{12}—C_{18} acids into the detergent range of surface activity. Curiously, the *I.G.* did not produce any synthetic detergents of this type but several, e.g. the *Ethofats*, are marketed in America. This difference between the two countries possibly arises from differences in compounding and methods of evaluation.

2. *Derivatives of alcohols*

These are ethers of the general formula

$$R\text{ -}O—(C_2H_4O—)_nH$$

Again, the wide range of primary and secondary alcohols from natural and synthetic sources already described can be used. *Leonil O*, a condensate of cetyl alcohol with 15 mol ethylene oxide was advocated by the *I.G.* as a detergent for wool, a feature being that it is not substantive to wool and hence effective at lower concentrations than the anionic types which are substantive. Products of similar character have been made from C_{18}—C_{21} alcohols derived from paraffin oxidation acids using the optimum amount of ethylene oxide as determined by experiment to give the required properties.[13] It should be noted here that the sulphates of such alcohols would probably be of little interest on account of poor solubility. An American product, Brij-35, is said to be derived from coconut oil alcohols.

3. *Derivatives of amides*

The chemistry of the reaction with acid amides has not been clarified. Theoretically, the following may occur:

$$R.CO.NH_2 + C_2H_4O \rightarrow R.CO.NH.C_2H_4OH$$

$$R.CO.NH.C_2H_4OH + C_2H_4O \begin{cases} \nearrow R.CO.NH.C_2H_4O.C_2H_4OH & (I) \\ \searrow R.CO.N(C_2H_4OH)_2 & (II) \end{cases}$$

(I) and (II) can then give rise to a further variety of products depending on which active hydrogen reacts and in all probability a complex mixture of products results. Products of this type have been marketed in the U.S.A. (*Ethomids*) and reported to be good detergents though here too, the *I.G.* did not make detergents of this character.

4. *Derivatives of amines*

Amines, like the amides, can in theory give rise to a wide variety of products but as diethanolamine derivatives are known to be produced in this manner from amines of low molecular weight it seems reasonable to expect that the polyethenoxy derivatives will be of the type

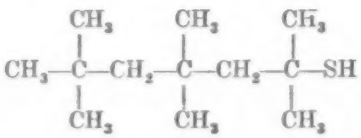
$$RN\begin{cases} (C_2H_4O-)_xH \\ (C_2H_4O-)_yH \end{cases}$$

Fatty amine derivatives of this nature are available in the U.S.A. as *Ethomeens*: they are of course cationic in character under acid conditions.

5. *Derivatives of mercaptans*

These are thio-ethers of the general type $R—S—(C_2H_4O—)_nH$.

The most economic source of mercaptans is by addition of H_2S to olefines such as tri*iso*butylene to give



which with 12 to 15 mols ethylene oxide gives detergents especially free from foaming.[163] *Nonic* 218, made in the U.S.A., is of this type.

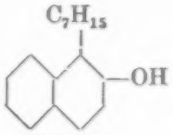
6. *Derivatives of phenols*

These are of the type R— —O—$(C_2H_4O—)_nH$ where the aromatic ring may carry other groups.

Alkyl phenols being available from cheap raw materials and their polyethenoxy derivatives being free from the hydrolytic defects inherent in the fatty acid derivatives, such condensates are probably the most widely used

non-ionic detergents. The alkyl groups may vary according to raw material availability and economics from such as two C_4—C_8 residues to one C_8—C_{16} residue. The alkyl groups may be introduced by normal academic methods e.g. by alkylating with alcohols or alkyl chlorides or by reduction of the corresponding ketones but the usual technical route is by alkylation with olefines. The *I.G.* used mainly di*iso*butylene, a C_6—C_7 olefine mixture obtained by dehydrating by-product C_6—C_7 alcohols from methanol synthesis, a dimer of this mixture, and propylene tetramer. The phenol may be phenol itself, the various cresols or mixtures of these, xylenols or naphthols. When introducing two alkyl groups the *I.G.* preferred to use phenol itself. More complex phenols such as alkylphenol-formaldehyde condensates have been used.[169]

The preparation of the alkyl phenols is carried out by normal methods of reacting the phenol and olefine in the presence of a suitable catalyst such as sulphuric acid. The *I.G.* used boron trifluoride except for alkyl naphthols where aluminium chloride was used in order to obtain the alkyl groups in the ring adjacent to that carrying the hydroxyl group, products of this structure (II) being said to be more effective than where the alkyl and hydroxy groups are in the same ring (I).



(I)

(II)

Condensation with ethylene oxide is effected in the normal manner and the amount introduced determined by the properties desired. By so regulating the amount of ethylene oxide, products having essentially the same detergent properties can be obtained from different intermediates and they can be specially designed to meet specific uses. Thus, the *I.G.* considered *Leonil O* (oleyl or cetyl alcohol—15 mol ethylene oxide), *Leonil FFO* (C_6—C_7 alkylnaphthol—8 mol ethylene oxide), *Emulphor STS* (a C_6—C_7 dialkylated phenol—7·5 mol ethylene oxide) and *Igepal W* (a tert. octyl phenol—6 mol ethylene oxide) substantially equivalent for wool detergency and they produced a dodecyl cresol—12 mol condensate for cotton detergency (*Igepal C* which could also be a tert. octylphenol—10 mol condensate) and a 10 mol condensate (*Igepal F*) for washing feathers. Similarly, products equivalent to the above can be obtained by condensing dodecyl "brenzoel" (a mixture of methyl, polymethyl and ethyl catechols from brown coal tar) with the requisite amount of ethylene oxide, 11 mol for a wool detergent (Ue 7/08) and higher (12 to 15 mol) for cotton.

Continuous processes for making these ethylene oxide condensates have been described.[13], [206]

Products of this type are also produced in the U.S.A. (e.g. *Triton NE*, *Antarox* 200) and in England (*Lissapol N*).

7. *Miscellaneous polyethenoxy products*

Just as ethylene oxide reacts with amides so also does it react with sulphonamides and here the *Reed* reaction is especially attractive as a source of raw materials.

$$R.H \rightarrow R.SO_2Cl \rightarrow R.SO_2NH_2 \rightarrow R.SO_2N\begin{matrix}(C_2H_4O-)_xH\\(C_2H_4O-)_yH\end{matrix}$$

Such products have been examined in Germany and where $R = C_{15}$ and $x + y = 5$ a moderately good detergent is obtained, but it is slightly inferior to *Igepals C* and *W*.

More recently a novel type of product (the *Pluronics*) has been marketed in the U.S.A. by the *Wyandotte Chemical Co.* in which the hydrophobic part of the molecule is built up with propenoxy residues. These are obtained[170] by reacting 1 : 2-propylene oxide with a limited amount of water or with 1 : 2-propylene glycol in the presence of caustic soda as catalyst. The lower polypropylene glycols are soluble in water but insoluble when the molecular weight is above about 900. These latter are used as the hydrophobic portion and solubility conferred by reaction with ethylene oxide to give products of the type

$$HO(C_2H_4O)_a(C_3H_6O)_b(C_2H_4O)_cH$$

The precise structure of the propenoxy chain is not given but judging from other reactions[171] of this material with hydroxyl groups it will presumably contain

$$-CH(CH_3)-CH_2-O-CH_2-CH(CH_3)-,\quad -CH_2-CH(CH_3)-O-CH(CH_3)-CH_2- \text{ and}$$

$$-CH_2-CH(CH_3)-O-CH_2-CH(CH_3)-$$

linkages. The quantity of ethylene oxide required depends on the molecular weight of the propenoxy base but is usually adequate at around 40 per cent by weight of the total condensate. Such products are liquid but by selection of the base and adding up to 70 per cent of ethylene oxide in the final product detergents which are sufficiently hard to be flaked can be obtained. The possibilities of wide variations in both groups permit products to be made to any desired design.

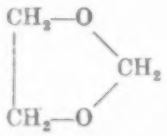
Fatty acyl hydrazides, glucosides, disulphimides, urethanes, aldehydes, ketones, nitriles, iminazolines are amongst the other types of intermediates suggested for reaction with ethylene oxide but in some cases the chemistry of the reaction occurring is obscure. In addition, of course, many of the types of intermediates used for anionic agents where the hydrophobic portion of the molecule is joined by some linkage such as ester, amide, ether, etc., to a residue carrying a reactive group such as hydroxyl, carboxy, amino, etc., can be used.

8. *Properties of the polyethenoxy detergents*

With the exception of the harder *Pluronic* type described above, the polyethenoxy products vary in physical properties from viscous liquids to soft waxes. They therefore do not lend themselves easily to formulation into powder forms but several ways of doing this have been described[172] and powder products are marketed, e.g. *All*, in the U.S.A. They are also marketed as 100 per cent agents or as aqueous solutions for general use in the household and in industry. Their relatively poor foaming power is a psychological defect for domestic use where the housewife has been for so long accustomed, and further conditioned by advertising, to associate cleansing with sudsing but this property is being exploited for use where foam can be a nuisance as in mechanical washing operations. The polyethenoxy chain is stable to alkalies and the common acids and relatively unaffected by electrolytes: weaknesses in particular types may exist in the linkage between the hydrophobic and hydrophilic residues as in the case of the anionic types, for example with ester-linkages. The characteristic property of decreasing solubility in water with increase of temperature has already been mentioned and is related to the number of ethenoxy groups present, the greater the number the higher the temperature at which this happens. Hydration presumably occurs by hydrogen bonding of water molecules to the ether oxygen atoms and such a weak bond would hardly be expected to have much thermal stability. They also form complexes with high-molecular polybasic acids, e.g. phosphotungstomolybdic acid and syntans and also with calcium chloride.

B. Miscellaneous non-ionic types

Closely related to the polyethenoxy products are those which can be obtained by reacting fatty acids and alcohols with 1 : 3-dioxolane



or with its precursors ethylene glycol and formaldehyde giving products of the type

$$R{-}O(CH_2{-}O{-}C_2H_4O{-})_nH$$[173]

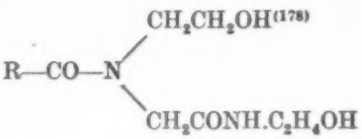
which on account of their acetal linkage would be expected to be unstable under mildly acid conditions.

Glycide, $CH_2OH.CH(O)CH_2$, can be used in place of ethylene oxide and polyglycide[174] or polyglycerols in place of polyglycols to produce detergents. Likewise, hydroxy ethers of polyhydric alcohols, e.g. sorbitol hexahydroxyethyl ether, can be partially esterified with fatty acids to give detergents.[175]

The reducing sugars have also been reacted with appropriate intermediates

to confer solubility, e.g. lauroylhydrazide with glucose.[176] Chloroalkylglucosides have been converted to ester and amino derivatives.[177]

Solubility can be introduced by a plurality of amide and hydroxy groups, for example by acylating β-hydroxyethylaminoacetamidoethanol with a fatty acid to give



A much simpler procedure[179] is to react the fatty acid with 2 mol of diethanolamine at 150 to 170°C until free fatty acid is 5 per cent or less and an interesting account of this is given by SCHWARTZ and PERRY.[2] The chemistry of the process has not been explained. Water is eliminated but both esterification and acylation reactions can occur. The product does not behave as a cationic detergent which suggests that the simple ester $R.COO.C_2H_4NH.C_2H_4OH$ is not formed but the formation of products containing both ester and amide groupings cannot be excluded. Pure diethanolamine can be distilled out of the product but the residue has inferior properties which can be restored not by mixing alone but by re-heating the components together at 120 to 140°C. This transformation occurs with no loss of water or change in weight. It is suggested that some physico-chemical change occurs and that the molecules are combined into oriented groupings or micelles which maintain their identity in dilute solution but there is an obvious need for a thorough investigation of the whole problem. Products of this type are marketed in the U.S.A., e.g. the *Ninols*, *Alrosols*, etc., for laundering textiles and for shampoos. Amines other than diethanolamine, e.g. trimethylolaminomethane (I) and diglycerylamine (II) can be used:

$NH_2C(CH_2OH)_3$ (I) $\qquad$ $NH(CH_2.CHOH.CH_2OH)_2$ (II)

Products from the latter are conveniently made via the corresponding ketals which are subsequently decomposed by hydrolysis:[180] presumably substances of more clearly defined constitution will be obtained in this way.

Betaines, inasmuch as they are electrically neutral, are classified by MOILLIET and COLLIE[3] as non-ionic agents though these authors stress that their amphoteric character should not be overlooked. They are available by the classical methods from long chain acids, halides, etc., e.g.:

$R.CH_2CO_2H \rightarrow R.CHBr.CO_2H \rightarrow R.CH\overset{+}{N}(Me)_3.CO_2^-$[181]

$ROCH_2Cl + Me_2NCH_2CO_2H \rightarrow ROCH_2\overset{+}{N}(Me_2)CH_2CO_2^-$[182]

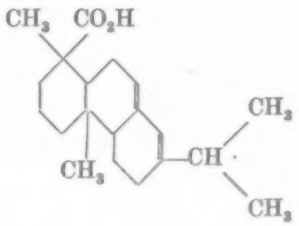
$R.NH_2 \rightarrow R.NMe_2 \rightarrow R.\overset{+}{N}(Me_2)CH_2CO_2^-$[183]

$C_{12}H_{25}NMe_2 + ClCH_2CHOHCH_2SO_3Na \rightarrow C_{12}H_{25}\overset{+}{N}(Me_2)CH_2CHOHCH_2SO_3^-$[184]

Many variations on this theme have been made, but although some of the products are good detergents under acid, neutral and alkaline conditions, they have not been developed technically for this purpose presumably on grounds of cost.

V. Miscellaneous Raw Materials

Intermediates other than the natural fatty esters, paraffins and olefines have been examined extensively for detergent outlets, anionic, non-ionic and cationic types coming into consideration. The naphthenic acids available as by-products from petroleum refining are frequently mentioned as alternatives to fatty acids and the derived alcohols as alternatives to the fatty alcohols in the reactions described for these products. The odour associated with them has probably been a deterrent to technical development. Likewise rosin acids and tall oil acids from the resin and wood pulp industries are of interest: ethylene oxide condensates of tall oil acids are marketed (e.g. *Sterox CD* and *Renex*) in the U.S.A. as non-ionic detergents. They are of course complex mixtures whose composition will depend on methods of production. The abietic acid of rosin has the probable structure:



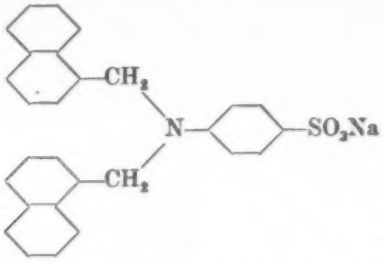
i.e. a dimethylisopropyldecahydrophenanthrene derivative. Tall oil acids contain in addition to rosin acids a variety of fatty acids and other products. Hydrogenated derivatives of abietic acid such as hydroabietic acid and perhydroabietyl alcohol are mentioned as alternatives to the fatty acids and fatty alcohols for producing anionic and non-ionic detergents. A condensate of hydroabietyl alcohols with 15 mols ethylene oxide is stated to be an effective detergent, production of which was under consideration in Germany.[9]

The oil from the cashew-nut shell can provide a mixture of unsaturated long-chain phenols known as cardanol[185] which can be used as such or reduced to tetrahydroanacardol (m-pentadecylphenol). These can be employed[136] as intermediates for detergents in lieu of the synthetic alkyl phenols already described, e.g. they may be sulphonated, etherified and sulphonated, or reacted with ethylene oxide.

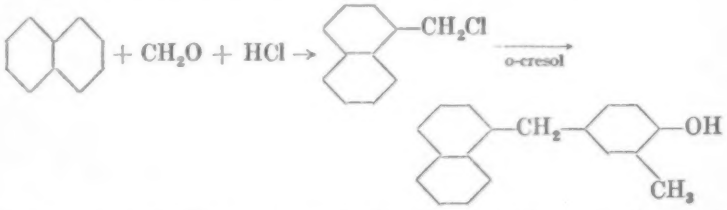
Terpenes and lecithin from vegetable sources and sterols from wool grease have also been mentioned.

Of greater interest chemically, however, is the use of aromatic hydrocarbons as intermediates, several such groupings being combined together and used in

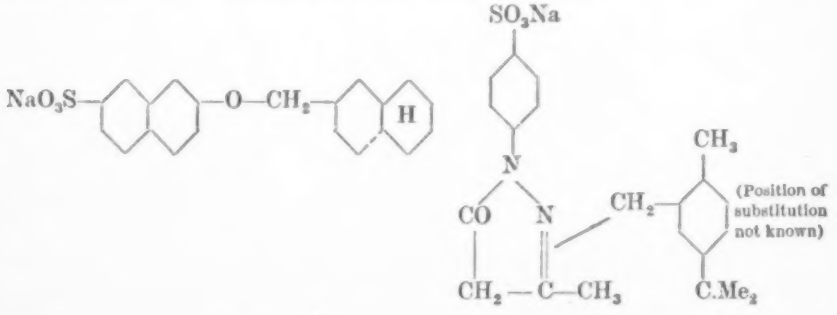
place of the usual aliphatic or alkylaromatic residues. Products such as sodium N : N-di-α-menaphthylsulphanilate:[187]

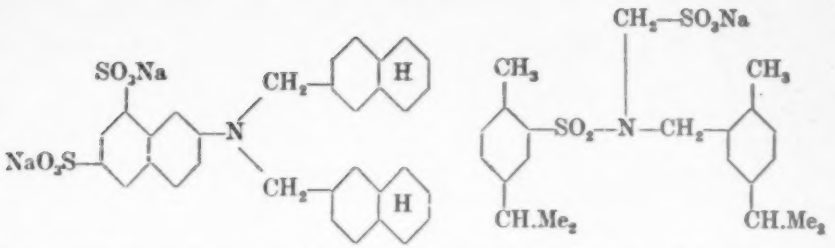


are stated to have cleansing properties but greater interest has apparently been found in partially or completely hydrogenated derivatives. Thus, compounds such as p-hydroxydiphenyl, p-benzylphenol and 1-benzyl-2-naphthol are hydrogenated to the secondary alcohols which are then converted to sulphoacetates, glycollic acid ethers, reacted with ethylene oxide and so on. Similarly menaphthylcresol from 1-chlormethylnaphthalene and o-cresol

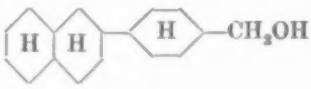


can be fully or partly hydrogenated, decahydronaphthalene can be chlorinated and the monochloro derivative reacted with phenol to give the decahydronaphthylphenol, the hydroxy groups being used to attach solubilising groups in ways already described.[188] Related products have been made starting from other aromatic and heterocyclic types such as anthracene, phenanthrene and carbazole. Chlormethylated aromatic derivatives such as are obtainable from tetralin and p-cymene have also been reacted[189] with aromatic derivatives carrying suitable reactive and solubilising groups, e.g. 2-hydroxynaphthalene-6-sulphonic acid, 2-aminonaphthalene-6 : 8-disulphonic acid and 1-p-sulphophenyl-3-methyl-5-pyrazolone giving substances such as:





Hydrocarbons such as cyclohexyltetralin give detergents on sulphonation.[190] More recently, perhydroarylcyclohexanols and the like have been dehydrated and the resultant cyclohexene derivative subjected to the *Oxo* reaction to give detergent intermediates of the type



for sulphation etc.[191]

VI. Detergent Adjuvants

Although many of the synthetic detergents are highly efficient when used alone, means of increasing this still further and of improving their properties in certain directions have been sought. Both inorganic and organic materials have been found of interest and have contributed enormously to the commercial success of synthetic detergents. An account of this has been given by Kramer.[192]

A. Inorganic salts

Mention has already been made of the effect of simple inorganic salts in increasing the detergency of the sulphated fatty alcohols. Flett[193] describes the effect of sodium sulphate on the detergency of the alkylaromatic sulphonates while according to Jordan *et al.*[194] a still further increase in detergency can be obtained by also adding a magnesium salt. The use of magnesium silicates as adjuvants has been patented[195] recently. Sodium silicates are commonly incorporated to prevent corrosion of metals and an account of this is given by Robinson[196]. Alkali carbonates and bicarbonates have been used but technical and commercial considerations have given rise to the extensive use of complex phosphates such as the pyrophosphates and tripolyphosphates, especially for heavy duty domestic laundering. These are normally employed as sodium salts but potassium tripolyphosphate is also of interest primarily because of higher solubility which permits compounding into liquid forms: advantages have also been claimed for acid triphosphates as builders.[197] Lithium salts have been claimed to improve the solubility of the monoalkyl sulphosuccinic ester type.[198]

B. Organic agents

The use of organic solvents as additions to detergents has long been a practice for specific purposes as is shown e.g. by the Laventin series of the *I.G.*[12] Of

much greater significance, however, is the more recent discovery in Germany that the addition of carboxymethyl ethers of cellulose, normally made by reaction of soda cellulose with chloracetic acid, prevents to a very great extent the re-disposition of soiling matter which frequently occurs when washing cottons with any type of synthetic detergent so that to-day this agent is a normal component of powders marketed for household use. Many other agents have been tried for this purpose e.g. sulphated cellulose and starch derivatives but carboxymethyl cellulose under its various trade names is still the preferred product. A survey of the use of carboxymethyl cellulose is given by POLLOK.[199]

Fatty amide derivatives such as the acyl derivatives from ethanolamine, morpholine etc. and coconut oil acids are claimed[200] to improve the detergent properties of sulphated and sulphonated types of detergents and also boost the detergency of the non-ionic polyethenoxy types.[201] The non-ionic types are also claimed to have a synergistic effect on the alkylaryl sulphonates.[202] Guanidine salts, e.g. the carbonate, are used to boost the detergency of alkylarylsulphonates.[203] Controlled amounts of alcohols may be added to fatty alcohol sulphates.[204] Other additives may be used for special purposes, e.g. ethylene thiourea to prevent discoloration of copper alloys, substances which fluoresce in the ultra-violet and so confer a bleaching effect, germicides and materials to minimize the de-fatting action of detergents on the human skin. An interesting example of this last type is the use of a substance, *Dermolan L*, a complex benzidine disulphonic acid derivative which functions as a mild tanning agent.[13], [209] Such additives and means of compounding into attractive physical forms can be of enormous commercial importance, especially when backed by the powers of advertising, though they are scarcely relevant to the chemistry of synthetic detergents as such.

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THE BIOCHEMISTRY OF FAT-SOLUBLE VITAMINS

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5

THE BIOCHEMISTRY OF FAT-SOLUBLE VITAMINS

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I. VITAMIN A

THIS vitamin is represented by several closely related compounds (or types of compounds) of which the following will be briefly mentioned as an introduction to the recent development of the biochemical aspects of the vitamin:

Vitamin A_1,
Vitamin A_2,
Neovitamin A,
Anhydrovitamin A,
Rehydrovitamin A,
Vitamin A aldehyde,
Vitamin A acid.

Regarding the study of the constitution and the synthesis of these vitamins A and their derivatives the reader is referred to the reviews of MILAS (1947), ZECHMEISTER (1949), ISLER (1950), VOGEL/KNOBLOCH (1950), DEUEL (1951), and BAXTER (1952). Detailed indication of the respective chemical and physical data are also found in several of these reviews.

Vitamin A_1 (usually called vitamin A) is the most widely distributed representative of the group. It occurs in all marine fish oils, and in the liver fat of most land vertebrates, chiefly in the form of esters with fatty acids. The geometrical structure of vitamin A_1 is all-*trans*. 0·344 μgr of vitamin A_1 acetate corresponding to 0·30 μgr of vitamin A_1 represents the international unit of vitamin A (World Health Organization, 1950).

Vitamin A_2 occurs in the liver of freshwater fish (EDISBURY, MORTON, and SIMPKINS, 1937), usually together with varying amounts of vitamin A_1. In marine fishes its amount is low. Freshwater fish can utilize vitamin A_1 but do not convert it to vitamin A_2 to any appreciable extent, if at all (MORCOS and SALAH, 1951). Vitamin A_2 may result from preformed vitamin A_2 in the diet or from the conversion of β-carotene and other provitamins to vitamin A_2. There is no direct evidence for the conversion of a specific provitamin A_2 (COLLINS and MORTON, 1953). According to FARRAR, HAMLET, HENBEST, and JONES (1951), and CAMA, DALVI, MORTON, and SALAH (1952), the evidence is very strong that vitamin A_2 is 3-dehydrovitamin A_1 (the numeral refers to carbon no. 3 in the ring).

The potency of vitamin A_2 in the rat growth test is 1,300,000 i.u. per gm (SHANTZ and BRINKMAN, 1950).

Neovitamin A has been found together with vitamin A_1 in the liver oil of several marine fishes in amounts up to about 35 per cent of the total vitamin A content (ROBESON and BAXTER, 1947). The potency in the rat growth test is about 80 per cent of the potency of vitamin A_1 (HARRIS, AMES, and BRINKMAN, 1951). ROBESON and BAXTER (1947) tentatively suggested, from spectrographic data, that neovitamin A has a *cis* configuration at the double bond at the CH_2 group nearest the hydroxyl group. According to the present mode of numbering the conjugated double bonds, beginning in the ring (cf. ZECHMEISTER, 1949) this means that neovitamin A should be 5-*cis*-vitamin A_1. WALD (1952) has proposed to designate neovitamin A (the 5-*cis* isomer) *neovitamin Aa*, while the designation *neovitamin Ab* is reserved for the 3-*cis* isomer.

Anhydrovitamin A (SHANTZ, CAWLEY, and EMBREE, 1943).

Anhydrovitamin A_1 and anhydrovitamin A_2 are formed by the action of mineral acids in an anhydrous solvent on vitamins A_1 and A_2, respectively. The anhydrovitamins A are hydrocarbons with 2 hydrogen and 1 oxygen atom less than the corresponding vitamins A. The position of the double bonds has not been definitely established. The potency in the rat growth test is 15,200 to 16,900 i.u. per gm anhydrovitamin A_1 (SHANTZ, CAWLEY, and EMBREE, 1943). Anhydrovitamin A has earlier been referred to as "cyclized vitamin A."

Rehydrovitamin A is formed from anhydrovitamin A when the latter is fed to rats (SHANTZ, 1950). Rehydrovitamin A is an isomer of vitamin A, perhaps with a shift in the position of the five conjugated double bonds. In the rat growth test it is about one-fifteenth as active as vitamin A.

Vitamin A aldehyde (retinene). Vitamin A aldehydes corresponding to vitamins A_1, A_2, and neovitamins A, are known. They can be produced *in vitro* by oxidation of the corresponding vitamin A with manganese dioxide (WALD, 1947–48; BALL, GOODWIN, and MORTON, 1948; CAMA, DALVI, MORTON, and SALAH, 1952; WALD, 1952; GRAHAM, VAN DORP, and ARENS, 1949). Vitamin A aldehyde can also be obtained by similar oxidation of carotene (MEUNIER, 1951). In the rat growth test vitamin A_1 aldehyde is approximately as active as vitamin A_1 (WENDLER, ROSENBLUM, and TISHLER, 1950). GLOVER, GOODWIN, and MORTON (1948) have shown that vitamin A_1 aldehyde is reduced to vitamin A_1 *in vivo*, irrespective of whether the administration takes place orally, subcutaneously or intraperitoneally. The retinenes are discussed further in connection with the role of vitamin A in vision.

Hydroxylated vitamin A aldehydes are formed when vitamin A is oxidized *in vitro* in various ways. Thus oxidation with permanganate in acid solution yields, besides vitamin A aldehyde, a "substance *Y*" having an absorption maximum in the $SbCl_3$ reaction at 560 mμ. Substance *Y* is 5,6-dihydroxy-vitamin A aldehyde (MEUNIER, MALLEIN, and JOUANNETEAU, 1949). Its activity in the rat growth test is between one-twentieth and one-twentyfifth of that of vitamin A (MEUNIER and FERRANDO, 1949).

Oxidation of vitamin A with vanadium oxide yields a mixture of two substances, Z_1 and Z_2 soluble in water and petroleum ether, respectively, and having an absorption maximum in the $SbCl_3$ reaction at 545 mμ. They are believed to be hydroxylated vitamin A aldehydes in equilibrium with each other, and the mixture is said to act antagonistic to vitamin A when fed to rats (cf. MEUNIER, 1952).

Vitamin A acid synthesized from β-ionylidene acetaldehyde (HEILBRON, JONES, LOWE, and WRIGHT, 1936) or from β-ionylidene crotonic acid (ARENS and VAN DORP, 1946) has been found to be as potent as vitamin A when given orally in the form of a solution of its sodium salt at *p*H 10 (VAN DORP and ARENS, 1946). Administered in other ways it was less active. It does not give rise to accumulation of vitamin A in the liver.

Kitol (EMBREE and SHANTZ, 1943). Kitol occurs primarily in whale oil, and to a lesser extent in fish oils. It has no vitamin A activity when ingested. However, it is converted into vitamin A (1 mol of vitamin A per mol Kitol destroyed) by heating *in vacuo* (molecular distillation). Kitol is believed to be a dihydric alcohol having a molecular weight about twice that of vitamin A.

(*a*) *Provitamins and their conversion into vitamin A*

A description of the chemistry and occurrence of the provitamins is outside the scope of this article. The reader is referred to the monographs of KARRER and JUCKER (1948), ZECHMEISTER (1949), DEUEL (1951), MACKINNEY (1952), INHOFFEN and SIEMER (1952), and GOODWIN (1952).

At least 10 different carotenoids exhibit provitamin A activity, viz. α- β- and γ-carotene, cryptoxanthin, β-carotene oxide, β-oxycarotene, semi-β-carotenone, aphanacene, aphanin and eichineone. β-carotene is the most potent. 0·6 μgm of β-carotene was formerly the international unit of vitamin A (League of Nations, Permanent Commission on Biological Standardization (1934)) and is still retained as a unit for provitamin A activity.

When the number of possible (partly already known) stereoisomers is taken into consideration, the total number of carotenoid provitamins A increases considerably. This aspect of the problem has been studied in recent years particularly by ZECHMEISTER, DEUEL, and their co-workers. They have shown that the crystalline carotenoids (mostly all-*trans*-forms), when brought into solution or melted, stereoisomerize into an equilibrium mixture of predominantly unchanged all-*trans* and various *cis*-compounds. The isomerization process is catalyzed by heat, light, iodine, and acid. The problem of stereoisomerism is further complicated by the advent of synthetic carotenoids (KARRER and EUGSTER, 1950).

The absorption maxima, in the visible light, of the *cis*-forms are found at shorter wavelengths than those of the *trans*-forms, and the extinction coefficient at these maxima is lower for the *cis*-forms. In the ultraviolet, the all-*trans* carotenoids have practically no maximum between 320 and 380 mμ,

but the *cis*-forms have a characteristic maximum, the "*cis*-peak" in this region (ZECHMEISTER and POLGAR, 1943).

Examples of *cis-trans* isomers of β-carotene

β-carotene	all-*trans*	usual crystalline form
neo-β-carotene *U*	3-mono-*cis*	produced artifically by isomerization
neo-β-carotene *V*	3,9-(or 5,7-) di-*cis*	
neo-β-carotene *B*	3,6-di-*cis*	

In the above-mentioned temporary nomenclature, the numbers refer to the double bonds, beginning with the double bond in the ionone ring. The prefix "neo" designates the artificial forms. *Cis*-forms which have been found in nature (e.g. one such of γ-carotene) are given the prefix "pro." Letters *A*, *B*, *C*, etc., refer to isomers which are less firmly adsorbed than the all-*trans*-form, being the one which is nearest below the all-*trans*-form in the chromatogram. *T*, *U*, *V* refer to more firmly adsorbed forms, *T* being the form located nearest above the all-*trans*-form. Exceptions to this rule are found, however. The nomenclature of the sterecisomers has been discussed and criticized by MACKINNEY (1952).

In the bioassay for vitamin A activity, the all-*trans*-form of a given carotenoid is usually found to be the most potent. An exception to this rule is found in the γ-carotene group, where pro-γ-carotene (about penta-*cis*) is at least as potent as all-*trans*-γ-carotene.

While the carotenoid provitamins A usually originate in green plants, certain observations seem to indicate that micro-organisms in the ileum and coecum of sheep may synthesize carotene (MCGILLIVRAY, 1951). The conversion of the carotenoid provitamins into vitamin A takes place in the intestinal wall. SEXTON, MEHL, and DEUEL (1946) showed that parenteral administration of carotene failed to correct vitamin A deficiency in rats, even though the carotene accumulated in the liver. Direct evidence of the role of the intestinal wall in the conversion of the provitamins A was given by GLOVER, GOODWIN, and MORTON (1947), MATTSON, MEHL, and DEUEL (1947), WIESE, MEHL, and DEUEL (1947), THOMPSON, GANGULY, and KON (1947), THOMPSON, COATES, and KON (1950), and ROSENBERG and SOBEL (1953). KRAUSE and PIERCE (1948) showed that oral ingestion of an aqueous dispersion of carotene in water (with "Tween 40" as solubilizing agent) resulted in a considerable increase of vitamin A in the blood even when the liver was blocked by ligature of the portal vein, the hepatic artery and the common bile duct. STALLCUP and HERMAN (1950) incubated small intestine, minced liver and blood plasma of calves with a colloidal carotene solution and found conversion to vitamin A in intestine and liver but not in plasma.

Retinene has been mentioned as a possible intermediate in the conversion of carotene to vitamin A (HUNTER, 1946). The site of conversion of cryptoxanthin to vitamin A in the rat was studied by PATEL, MEHL, and DEUEL (1951) and

found to be the same as for carotene. WITH (1946) has postulated that in chicks cryptoxanthin may act as vitamin A without being converted.

(*b*) *Absorption, utilization and deposition*

The degree of utilization of a given amount of ingested *provitamin A* is determined by:

1. The nature of the provitamin,
2. Factors which influence the absorption from the intestine,
3. Factors which promote or inhibit destruction of the provitamin or of vitamin A,
4. The species of experimental animal.

In case of β-carotene the degree of utilization in the usual rat growth test is less than 50 per cent of the stoichiometrically possible, since, according to the new standards, 0·6 μgm of carotene gives the same growth response as 0·3 μgm of vitamin A alcohol fed as the acetate.

Vitamin A alcohol ingested as such or arising from hydrolysis of ingested vitamin A esters in the intestine is esterified in the intestinal wall during absorption (EDEN and SELLERS, 1950). Feeding of carotene also results in a flow of vitamin A esters into the circulation. Vitamin A (from carotene or preformed) is absorbed via the intestinal lymph, not through the portal blood of pigs and rats (THOMPSON, GANGULY, and KON, 1949) and of the guinea pig (WOYTKIW and ESSELBAUGH, 1951).

The velocity of absorption of ingested vitamin A depends on whether the vitamin is given in oil solution or as an aqueous colloidal dispersion, the latter form being more rapidly absorbed (KRAMER, SOBEL, and GOTTFRIED, 1947). In aqueous carrier the natural ester and the acetate are better utilized than the alcohol (liver storage in chicks being used as the basis for comparison (MARCH, ENGLISH, and BIELY, 1952)).

A comparison of the utilization of synthetic and natural vitamin A in margarine was made by AMES, LUDWIG, SWANSON, and HARRIS (1952). They found no difference between the two forms of vitamin A regarding growth, reproduction and lactation of rats through three generations, under their experimental conditions.

Ingested vitamin A is not only deposited in the liver, but also, to a considerable extent, in the kidney, especially at low levels of intake (EDEN and MOORE, 1950, 1951). Liver homogenates are able to hydrolyze vitamin A acetate but not the palmitate or other "natural esters." Unlike homogenates of intestine and kidney, liver homogenates are unable to esterify vitamin A (KRAUSE and POWELL, 1953). In the plasma, vitamin A alcohol has been found to be associated with the more soluble fraction of the proteins, and vitamin A esters with the least soluble fraction (GANGULY, KRINSKY, MEHL, and DEUEL, 1952). GANGULY and KRINSKY (1953) found no correlation between vitamin A alcohol levels in plasma and liver in rats. In the normal distribution of vitamin A of the rat liver cell, fraction *X* (liver homogenate minus nuclei and mitochondria) seems to contain the highest concentration,

the nuclei the least (Powell and Krause, 1953). Krinsky and Ganguly (1953) have studied the distribution of vitamin A ester and vitamin A alcohol in the rat liver. They found the mitochondria and nuclei essentially free of vitamin A. Vitamin A ester was mostly found in the centripetally migrating layer, while vitamin A alcohol was also present in the microsomes and the supernatant layer. They suggest that vitamin A ester and vitamin A alcohol are associated with different proteins during storage within the liver cells.

The favourable influence of fat on absorption through the action as a carrier is more marked regarding carotene than regarding preformed vitamin A (Russel, Taylor, Walker, and Polskin, 1942). The nature of the dietary fat is of great importance: highly unsaturated fatty acids will tend to destroy vitamin A and its provitamins unless a sufficient amount of tocopherols or other antioxidants is present. This question is discussed further in the chapter on vitamin E. Mineral oil will decrease the absorption especially of the esters of vitamin A (Week and Sevigne, 1950). The same investigators found that ethyl laurate resulted in poor utilization of vitamin A alcohol and esters. Corn oil favoured the utilization of all forms, especially of the esters. According to Week and Sevigne (1950) vitamin A alcohol produces a greater response in serum vitamin A levels than does the acetate, when the substances are given in margarine.

Deuel, Greenberg, Savage, and Melnick (1951) compared the growth promoting potencies of vitamin A and carotene when given to vitamin A deficient rats, in cottonseed oil and in margarine. Carotene was particularly well utilized when fed in margarine. The feeding of xanthophylls and chlorophylls together with carotene reduce the utilization of the latter for liver storage to some extent (Kemmerer, Fraps, and DeMottier, 1947; Vavich and Kemmerer, 1950). A decrease in the utilization of β-carotene and preformed vitamin A by the feeding of large amounts of xanthophyll was also found by Kelley and Day (1950). The availability, to the rat, of the provitamins in yellow corn has recently been studied by Callison, Hallman, Martin, and Orent-Keiles (1953). Eighty-two per cent of the provitamins proper were found to be utilized as vitamin A.

The transfer of vitamin A to milk, in dairy cows, is particularly fast when the vitamin is given as an aqueous dispersion. The fastest transfer is obtained when the aqueous dispersion is injected intravenously (Sobel, Rosenberg, and Engel, 1952). This is taken as support of the diffusion theory.

Truscott (1953) has shown that the previously known increase in liver vitamin A storage in mice following castration is not due to the slightly increased food intake.

(c) *Determination*

Directions for the spectrophotometric determination at the absorption maximum at 325 mμ, using the correction of Morton and Stubbs (1948), are contained in the Technical Report Series No. 3 of the World Health Organization, Report of the Subcommittee on Fat-soluble Vitamins (1950). The

Pharmacopoeia of the United States XIV (1950) makes use of the spectrophotometric method according to the same principle as well as of the rat growth test and the reaction with antimony trichloride, the latter as a means of identification. Comments on the spectrophotometric method adopted by the W.H.O. have appeared by BRUNIUS (1950) and HJARDE (1950). HJARDE recommends chromatography instead of the correction for irrelevant absorption according to MORTON and STUBBS (1948). BRUNIUS emphasizes the necessity of identification. The Scandinavian Pharmacopoeia Council (1953) has adopted a method making use of chromatography as well as of the correction according to MORTON and STUBBS.

The colorimetric estimation of vitamin A in margarine has received special attention by MELNICK, LUCKMANN, and VAHLTEICH (1952), LUCKMANN, MELNICK, and VAHLTEICH (1952a and 1952b) and LUCKMANN, GOODING, and MELNICK (1952).

Various forms of the *rat growth test* have been described by BLISS and GYÖRGY in P. GYÖRGY: *Vitamin Methods* (1951). It is of interest that certain authors have increased the amount of vitamin E in the basal vitamin A-free diet by the addition of α-tocopherol. The U.S.P. diet contains 5 per cent cottonseed oil which gives an estimated amount of total tocopherol of about 5 mgm per 100 gm diet, and, at the same time, a considerable amount of linoleic acid (about 2 per cent). MURRAY and CAMPBELL (1952) have compared the physical-chemical methods with the rat growth test and the rat vaginal smear test of PUGSLEY, WILLS, and CRANDALL (1944). With pharmaceutical vitamin A preparations and with more or less oxidized cod liver oils they found good agreement between the spectrophotometric method with the correction of MORTON and STUBBS, and the biological methods. With cod liver oil oxidized to such an extent that the absorption maximum was shifted from 325 mμ the spectrophotometric and colorimetric methods gave results outside the confidence limits ($P = 0{\cdot}05$) of the bioassay.

(*d*) *Role of vitamin A in vision*

(i) ROLE IN VISION IN DIM LIGHT (SCOTOPIC VISION)

The association of vitamin A deficiency with nightblindness has been known since 1925, when FRIDERICIA and HOLM (1926) and HOLM (1925) demonstrated that regeneration of visual purple is impeded in rats deprived of vitamin A. Since then the relation between carotenoids and visual purple has been studied by several groups of investigators, for instance, in the laboratories of HECHT, LYTHGOE, MORTON, WALD, and others. Recently this research has culminated with the demonstration by WALD and co-workers, of the role played by *cis-trans* isomers of vitamin A and its aldehyde.

The following is a brief outline of the present state of knowledge.

The pigment of the rods, visual purple, is a chromoprotein consisting of the protein component *opsin* and the prosthetic group *retinene*. Retinene is

vitamin A aldehyde (MORTON and GOODWIN, 1944). In marine fishes and in land animals the visual purple is mainly *rhodopsin* which contains retinene$_1$ (vitamin A_1 aldehyde). In freshwater fish the visual purple is mainly *porphyropsin* which contains retinene$_2$ (vitamin A_2 aldehyde).* Visual purple is *bleached* in light through intermediary products to opsin + all-*trans*-retinene. The latter is reduced to all-*trans*-vitamin A, part of which passes into the blood circulation and undergoes metabolism (HUBBARD and WALD, 1952).

The existence of intermediary products in the bleaching process has been studied by LYTHGOE and co-workers (LYTHGOE, 1937; LYTHGOE and QUILLIAM, 1938). They found as a primary intermediate "*transient orange*" which is formed in light and subsequently changed (without the influence of light) into "*indicator yellow.*" LYTHGOE assumed transient orange and indicator yellow to be stages in the separation of carotenoid from protein in the rhodopsin molecule (1942).

The isolation of the light reaction from the reactions which do not require light was first carried out by BRODA and GODEVE (1941) by irradiating rhodopsin at minus 70°C. At this low temperature further changes of the primary product hardly occur. In WALD's nomenclature the primary irradiation product which can be obtained at temperatures between minus 40 and minus 100°C is *lumirhodopsin*. On warming, in light or darkness, to temperatures above minus 20°C lumirhodopsin is changed into *metarhodopsin*. The further change of metarhodopsin to opsin and retinene occurs only in the presence of water. In the *regeneration* of rhodopsin (the process which underlies adaptation for vision in dim light) retinene must be present as neoretinene b (the aldehyde corresponding to the alcohol neovitamin Ab), since this is the only form of retinene which will combine with opsin to form rhodopsin (HUBBARD and WALD, 1952). Dietary all-*trans* or other forms of vitamin A, are supposed to isomerize to a sufficient extent in the body to supply the necessary amount of neovitamin Ab (HUBBARD and WALD, 1952).

The binding of retinene to opsin is a bimolecular process when both components are present in equal amounts. When opsin is present in large excess, the process may be monomolecular. The process does not require energy (HUBBARD and WALD, 1952). The binding of retinene with amino groups in a protein carrier has been considered by COLLINS and MORTON (1950) and by WALD (1951). However, sulphydryl groups seem to be more important in the binding of retinene to opsin. WALD and BROWN (1951, 1952) have shown that retinene combines easier with sulphydryl groups than with amino groups at *p*H 6 which is optimal for the condensation of retinene with opsin. The binding of retinene to opsin is inhibited by *p*-chlormercuribenzoate, an effect which can be counteracted by glutathione. The fact that formaldehyde

* The designation rhodopsin for visual purple was used by KÜHNE (1879). KÜHNE and SEWALL (1880) were aware of a difference in colour of the retinas from fresh water fish (violet), and those of marine fish and most land animals (pink). WALD (1937a) proposed to call the pigment having an absorption maximum at 522 to 525 mμ, characteristic of fresh water fish, Porphyropsin, and to restrict the term rhodopsin to the visual purple with absorption maximum 500 mμ, characteristic of marine fish and most land animals.

also inhibits can be explained on the basis of SH groups as well as of NH_2 groups.

An explanation of the colour of opsin on the basis of halochromic structure has been discussed by COLLINS and MORTON (1950) and by HUBBARD and WALD (1952). The latter authors have made an interesting comparison with the blue colour given by vitamin A derivatives with antimony trichloride. According to MEUNIER and VINET (1947) this reaction gives rise to the formation of an all-*trans* product irrespective of the stereoisomer of vitamin A which enters the reaction.

The reduction of all-*trans*-retinene to all-*trans*-vitamin A takes place by means of alcohol dehydrogenase and cozymase. The same applies to the dehydrogenation of neovitamin Ab to neoretinene b (HUBBARD and WALD, 1952; BLISS, 1949, 1951).

There may be a short cut from all-*trans*-retinene to neoretinene b, since blue or violet light, heat, etc., catalyze the interconversion of these substances *in vitro*. However, it is believed that, *in vivo*, the intensity of the isomerizing blue and violet light is too low to play an important role in scotopic vision. The effect of heat is also considered to be of minor importance *in vivo*. Therefore, the possibility of the existence of a stereoisomerizing enzyme system is being considered. In laboratory work with the above-mentioned processes *in vitro* it is highly important to be aware of the isomerizing influence of blue light.

In visual purple the *cis*-configuration of retinene is supposed to be retained in accordance with the existence of a β band at about 350 mμ which is assumed to be characteristic of a prosthetic group with *cis*-configuration.

All the above-mentioned processes have been carried out *in vitro* mainly with spectrophotometry as qualitative criteria as well as means of quantitative measurements. An aqueous digitonin solution is used for the extraction of rhodopsin from the retina or from the outer segments of the rods isolated by a special flotation technique. Digitonin is also used for bringing the fat-soluble retinenes into an aqueous colloidal state in which they can react with opsin. The *in vitro* studies are complicated by the formation of by-products such as isoretinenes a and b and isorhodopsin, which substances are now held to be artifacts (HUBBARD and WALD, 1952).

As to the way in which the chemical processes of the rhodopsin cycle may give rise to nerve impulses, several of its steps have been considered. WALD and BROWN (1951) have focused attention on the liberation of SH-groups in opsin through the detachment of retinene.

In species having porphyropsin as the main component of their visual purple the bleaching and regeneration cycle is assumed to be similar to that described above. When rats are depleted of vitamin A and fed vitamin A_2, their retinas take up this latter vitamin and then contain porphyropsin instead of rhodopsin, as shown by the spectral curves of the retinal pigment (shift of the absorption maximum from 500 mμ to 520 mμ). However, there seems to be a tendency to retain retinene$_1$ in the visual purple as long as any is available (SHANTZ, EMBREE, HODGE, and WILLS, 1946).

(ii) ROLE IN VISION IN BRIGHT LIGHT (PHOTOPIC VISION)

Complete blindness is not known as a symptom of vitamin A deficiency. However, an increase in the threshold value for cone vision paralleling the increase in the threshold for rod vision in young men receiving a vitamin A deficient diet has been reported by HECHT and MANDELBAUM (1938).

WALD (1937b) extracted a pigment iodopsin from chicken retinas together with rhodopsin from the rods. Iodopsin which arises from the cones has been studied further by WALD, BROWN, and SMITH (1952). Its maximal absorption is at 562 mμ while the band of rhodopsin is at 500 mμ. This explains why the spectral sensitivity of the eye shifts towards the red as one goes from dim to bright light, that is: from rod to cone vision. Iodopsin bleaches in red light which does not bleach rhodopsin. Bleaching of iodopsin results in an inactive retinene, apparently all-*trans*-retinene, and a protein which is different from opsin. The protein component of iodopsin has been called *photopsin* to distinguish it from the opsin (scotopsin) from the rods. The all-*trans*-retinene is reduced to all-*trans* vitamin A by the same enzyme system as in the rods. The synthesis of iodopsin requires an active *cis*-isomer of retinene, the same as is needed for the synthesis of rhodopsin. For cone vision to continue, therefore, this active isomer or its corresponding vitamin A must be supplied, either from the circulation or from isomerization of the inactive form.

Regarding trichromatic vision in man less information is available. HECHT (1931) suggested that the cones might contain three distinct photo-labile pigments differing in absorption characteristics. WALD (1937) found in the chicken retina three groups of colour filters which may provide a possible basis for hue differentiation with only one photo-labile pigment, iodopsin. These colour filters are lacking in mammals, and here it is possible that colour vision is probably mediated by a number of different light sensitive pigments distributed among the cones (WALD, 1953a).

A new visual pigment, Cyanopsin, has been synthesized by WALD, BROWN, and SMITH (1953) by combining chicken cone opsin with retinene$_2$. It is expected to occur in any retina which contains vitamin A$_2$ or retinene$_2$ and cone opsin.

A review of the biochemistry of vision has been given by WALD (1953b).

(*e*) *Vitamin A deficiency symptoms*

It is not the place here to list the various long-known symptoms of vitamin A deficiency. It might be worthwhile, however, to call attention to one aspect, viz. bone dysplasia with secondary nervous symptoms such as deafness, of which a thorough review has been given recently by MELLANBY (1950).

The symptoms of vitamin A deficiency have been subjected to a renewed study by LOWE, MORTON, and HARRISON (1953) with special attention to (*a*) the depletion process, (*b*) the specificity of vitamin A, (*c*) liver lipid unsaponifiable matter, (*d*) xerophthalmic eye tissue, (*e*) abnormal constituents of other liver lipids, (*f*) cholesterol-vitamin A interactions, (*g*) vitamin A depletion and the adrenal cortex

(*f*) *Requirements and recommended allowances*

The U.S. National Research Council, Committee on Animal Nutrition, has issued tables of Recommended Allowances for Domestic Animals (1944–49), in which a certain margin of safety has been added to the assessed requirements.

ALMQUIST (1953) has recently undertaken an evaluation of the data from the literature concerning the vitamin A requirement of chicks and poults. He estimates the minimum requirement for growth of chicks at approximately 100 i.u., and that for poults at 2300 i.u. per lb. of diet. A review of the vitamin A requirement of poultry is contained in a paper by HOGAN (1950).

The vitamin A requirement of the mouse has been estimated by MCCARTHY and CERECEDO (1953) at 1 i.u. per day (vitamin A acetate in cottonseed oil). This amount gives good growth, normal haematology and normal reproduction function of the male, which function is particularly sensitive to a deficiency of vitamin A. Since storage of vitamin A also occurs with 1 i.u. per day in the adult as well as during the period of rapid growth, this amount is believed to be somewhat in excess of the needs of the growing mouse.

For the rat the vitamin A requirement has been assessed, through figures from the literature, to be 40 i.u. per 100 gm of diet (BROWN and STURTEVANT, 1949).

The U.S. National Research Council, Food and Nutrition Board, has recommended a daily allowance of 5000 i.u. of vitamin A for normal adult persons, based on the premise that approximately two-thirds of the vitamin A value is contributed by carotene and that carotene has half or less than half the value of vitamin A (1948). The minimum daily requirement is considered, by the U.S. Food and Drug Administration, to be 4000 i.u. for normal adults (1941).

It is not known in detail how far the requirement for vitamin A as an essential nutrient is distributed throughout the animal kingdom. It is certain that at least all species which depend upon rhodopsin, porphyropsin, iodopsin or the like for the visual processes must receive vitamin A in the diet for normal physiological functions. This means that all vertebrates and some invertebrates require vitamin A. Among the invertebrates the squid, *Loligo pealii*, has been shown to possess rhodopsin (ST. GEORGE and WALD, 1949). Certain insects, such as the cockroach, *Blatella germanica*, could be raised through several generations on a vitamin A deficient rat diet, and the oil extracted from them did not contain vitamin A (MCCAY, 1938; BROWERS and MCCAY, 1940). The clothes moth, *Tineola biselliella*, does not require vitamin A either (CROWELL and MCCAY, 1937).

GLOVER, MORTON, and ROSEN (1952) examined developing salmon eggs, over a period of about 3 months from fertilization, and found a marked conservation of astaxanthin and a striking lack of evidence that vitamin A (or A_2) is required in more than traces, if at all.

(g) *Hypervitaminosis A*

Hypervitaminosis A gained renewed interest when RODAHL and MOORE (1943) found that the toxicity of bear and seal liver is related to an extraordinary high content of vitamin A. This study was followed up by MOORE, and WANG (1945), RODAHL, (1949a,b,c, 1950a,b), WALKER, EYLENBURG and MOORE (1947) and LIGHT, ALSCHER, and FREY (1944). HERBST, PAVCEK, and ELVEHJEM (1944) found similar effect of telang liver which also contain an extremely high amount of vitamin A.

According to RODAHL (1950a) the symptoms, in rats, of acute intoxication are: signs of general malady, and unwell-being with changes in the pelts, drowsiness, weakness and decreased activity. These symptoms may well be compared with those observed in man and dogs following a single meal of bear or seal liver. The symptoms of chronic intoxication are: reduced weight gain, limping, stiffness in the limbs, alopecia, soreness and bleeding in the skin, exophthalmus, loss of hair and soreness around the eyes, as well as swelling of the palpebrae, and finally fractures of bones.

The skeletal changes in hypervitaminosis A are considered so specific that they have been proposed as a means of biological identification of vitamin A in concentrated preparations, available in sufficient quantity. This test can be carried out with rats on a stock diet, and only requires a relatively short test period (WOLBACH and MADDOCK, 1951). Studies on the effect of an excess of vitamin A on bone development, especially as found in foetal bone cultures, have been summarized in Nutrition Reviews (1952).

The bleeding tendency in hypervitaminosis A is partly due to decreased prothrombin activity which may be corrected by moderate doses of vitamin K (LIGHT, ALSCHER, and FREY, 1944; WALKER, EYLENBURG, and MOORE, 1947). This part of the symptom is discussed in the chapter: Vitamin K. An abnormal permeability or fragility of the capillaries seems also to be involved, but this anomality cannot be corrected by ascorbic acid (MOORE and WANG, 1945; WALKER, EYLENBURG, and MOORE, 1947).

Slight toxic symptoms can be produced in rats with daily doses of 50 i.u. per gm body weight. Two hundred to 500 i.u. per gm body weight cause severe symptoms and doses over 800 i.u. per gm body weight are lethal in less than a month (RODAHL, 1950b).

AMES, SWANSON, RISLEY, and HARRIS (1952) found that single dose feeding tests with vitamin A acetate in cottonseed oil indicated an LD_{50} of about 1 million units per gm body weight of the rat. The liver reached maximum vitamin A storage at about 50,000 units per gm of fresh liver tissue. At this level of storage the extractable liver oil had a potency of about 1 million units per gm.

When high doses of vitamin A (35,000 i.u.) were given to pregnant rats from the 2nd, 3rd or 4th to the 16th day of gestation, COHLAN (1953) found, in addition to a reduction of the number of litters carried to term, characteristic congenital anomalies, such as extrusion of the brain to the external surface of

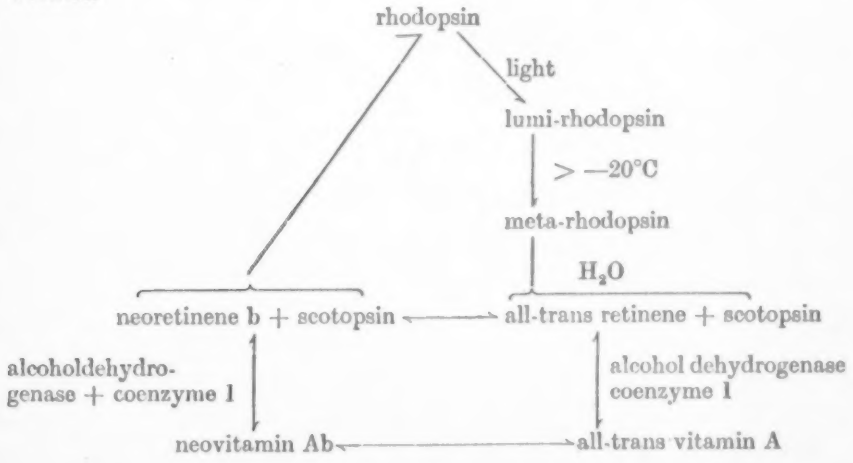
the head, and, less frequently, macroglossia, harelip, cleft palate, and gross defects in eye development.

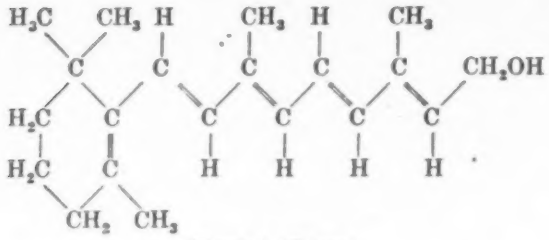
A case of hypervitaminosis A in a 3-year-old child due to excessive medication was described by JOSEPHS (1944). Other observations of the consequences of extreme medication with vitamin A are listed by RODAHL (1950a). In JOSEPHS' case, 240,000 U.S.P. units of vitamin A were given daily in the form of halibut liver oil from the age of between 2 and 3 months up to 3 years. The literature contains several partly conflicting statements about the tolerance of high doses of vitamin A given to children or adults through shorter periods (cf. RODAHL, 1950a). All clinical observations of hypervitaminosis based on experiments with fish oil must be considered with a view to the content of vitamin D as well as of A.

(*h*) "*Lard factor*"

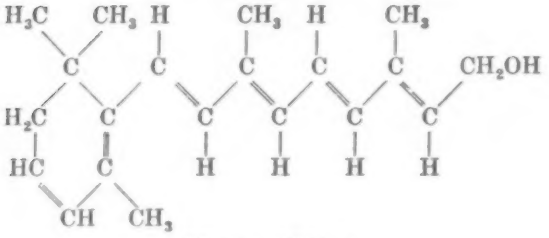
KAUNITZ and SLANETZ (1950, 1951) announced the existence of a factor in lard which would prevent vitamin A deficiency symptoms in rats without responding to the chemical reactions for vitamin A. The factor occurs in the fraction which distils from lard at 214°C and 0·001 mg Hg pressure. However, ENGEL (1951) was unable to find any vitamin A activity of lard when this fat was included in a vitamin A deficient diet for rats at the level of 10 per cent. LOWE and MORTON (1953) confirmed the existence of a vitamin A active factor in lard which they were unable to determine by spectrophotometric or colorimetric methods. Nearly simultaneously, HERB, RIEMENSCHNEIDER, KAUNITZ, and SLANETZ (1953) announced that they had succeeded after chromatographic fractionation of unsaponifiables from lard to demonstrate the presence of vitamin A by the Carr Price reaction and the spectral curve. Therefore the lard factor is believed to be vitamin A which is difficult to determine chemically without thorough purification.

Bleaching and regeneration of visual purple, according to WALD and co-workers.

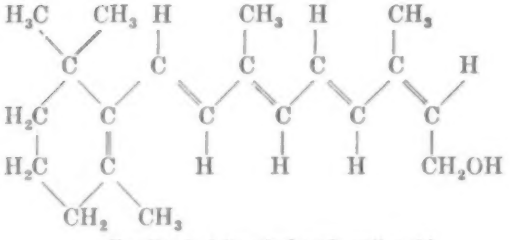




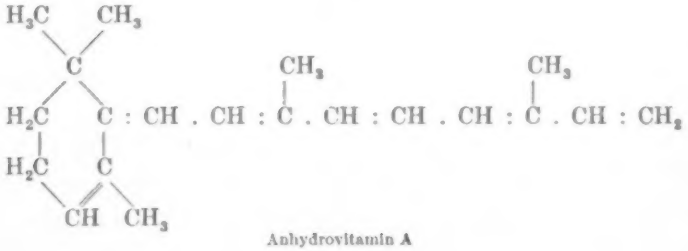
Vitamin A (all trans)



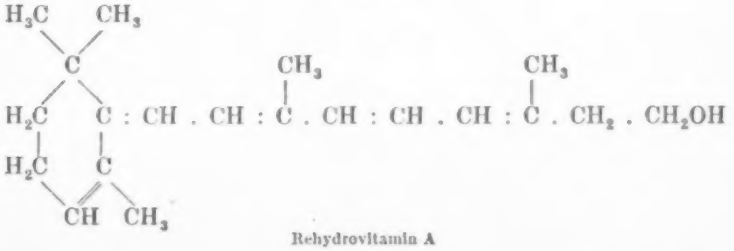
Vitamin A_2 (all trans)



Neovitamin A (terminal configuration cis)



Anhydrovitamin A



Rehydrovitamin A

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II. VITAMIN D

(*a*) *Representatives of the vitamin D group*

The chemistry of various forms of this vitamin and the corresponding provitamins has already been reviewed in vol. 1 of *Chemistry of Fats and other Lipids* by BERGMAN (1952). Other recent reviews on this aspect of vitamin D have been published by DEUEL (1951) and VOGEL-KNOBLOCH (1950).

Therefore, the brief renumeration shown in Table 1 may suffice as an introduction to the present subject.

The provitamin from the ribbed mussel *Modiolus demissus* (Dillwyn) called D_m was isolated by PETERING and WADDELL (1951). It is believed to be a C_{29} sterol. It seems to be equally active for rats and chicks (ROSENBERG and WADDELL, 1951).

(*b*) *Modes of activation of the provitamins D*

The usual procedure for converting the provitamins D into the vitamins is suitable irradiation in the ultraviolet and subsequent fractionation. In the activation of ergosterol the sequence of reactions is: ergosterol → lumisterol$_2$ → tachysterol$_2$ → vitamin D_2. Further irradiation of vitamin D_2 leads to suprasterol$_2$ I + suprasterol$_2$ II. A toxisterol$_2$ is also formed as a by-product of the irradiation process. Regarding the details the reader is referred to the aforementioned reviews.

D-vitamins may also be formed from sterols by purely chemical procedures. The first report to this effect was that of BILLS and MCDONALD (1926) who found that cholesterol heated in various organic solvents with floridin was

Table 1. Provitamins and vitamins D and their activities

Provitamin	*Vitamin*	*I.U./g for rats, in millions*	*Efficiency for chicks*
	D_1 = adduct of D_2 and lumisterol[a]		
ergosterol	D_2 (calciferol, ergocalciferol)	40[1]	slight[2]
7-dehydrocholesterol	D_3 (cholecalciferol)	40[3]	100%[4]
22-dihydroergosterol . .	D_4	12,5–15,5[5]	less than for rats[6]
7-dehydrositosterol . .	D_5	about 0,5[7]	less than for rats[8]
7-dehydrostigmasterol . .	D_6(?)	about 0,1[9]	?
7-dehydrocampestrol[10] . .			
epi-7-dehydrocholesterol[11] .		4 (?)	
5,7-norcholestadiene-3-β-ol[12] .			
5,7-androstadiene-3,17-diol .		0,1 (?)[13]	
3-hydroxy-5,7-choladienic acid		0,1 (?)[14]	

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[14] HASLEWOOD, G. A. D.; *Biochem. J.* 33 (1939) 454.

converted to a mixture of substances comprising one (not isolated) with antirachitic properties. This subject has received renewed attention by RAOUL and co-workers (1949–1952). These authors prepare substances with high vitamin D activity from cholesterol via 2-4, 2'-4'-bicholestadiene, a compound which can be obtained, for instance, through the Salkowski reaction or by heating with the Liebermann-Burchard reagent. This intermediary product is converted, with or without irradiation as a part of the procedure, into vitamin D active compounds, for which the authors reserve the designation vitamin D_7. (The term vitamin D_7 is used in VOGEL-KNOBLOCH's reference book to designate the vitamin D obtained by irradiation of the provitamin 7-dehydrocampesterol described by RUIGH (1942).) RAOUL and co-workers (1952b) report to have prepared their vitamin D_7 from fish oil, where it seems to be present besides vitamin D_3. The formation of vitamin D_3 from cholesterol without irradiation (treatment in ionizing millieu) has been described by RAOUL and co-workers (1949).

(c) *Observations on the origin of vitamin D in fish and the provitamin in rats*

Regarding the origin of vitamin D in fish, BILLS (1927) showed that vitamin D in eat fish oil was not increased by irradiating the fish, and it was not decreased by keeping the fish for 6 months in the dark on a vitamin D deficient diet. BILLS suggested that vitamin D may be synthesized by fish. Provitamin D_3

(7-dehydrocholesterol) is believed to be formed by enzymatic dehydrogenation of cholesterol in the intestinal wall of the rat (GLOVER, GLOVER, and MORTON, 1952).

(*d*) *Location of the provitamin in chicks and rats*

In chicks the sterols of the feet have a relatively high concentration of provitamin D while the secretion of preen gland is of little importance in this respect (ROSENBERG, 1953). In rats, WARD and MOORE (1953) have found a high concentration of a 7-dehydrosterol in the epididymis and preputial glands of the male and in the bulbi vestibuli of the female.

(*e*) *Standards*

The international unit of vitamin D activity is now represented by 0·025 μg of pure vitamin D_3 (World Health Organization, 1950). Formerly the unit was defined by the same amount of vitamin D_2 (League of Nations, 1935). The new unit (0·025 μgm vitamin D_3 is adopted by the United States Pharmacopoeia XIV (1950), and by the Association of Official Agricultural Chemists (1952). This eliminates complications arising from the presence of other vitamin D active substances besides vitamin D_3 in the formerly used reference oil (IRVIN, 1950).

(*f*) *Mode of action*

Children will get rickets in the absence of vitamin D, even with an optimal supply of calcium and phosphate in the diet. In young rats, however, rickets can only be produced when the diet contains an unfavourable ratio between calcium and phosphorus, usually high calcium, low phosphorus. Vitamin D aids the absorption of calcium and phosphate from the intestine, but it also seems to exert a direct influence on the deposition of calcium phosphate in the cartilage of the metaphysis. Isotope studies have confirmed the effect on calcium absorption (GREENBERG, 1945) and phosphorus retention in the bones (SHIMOTORI and MORGAN, 1943).

In rickets, serum phosphate is usually low, and serum (alkaline) phosphatase is high. The latter abnormality is generally considered to be due to a leakage of the phosphatase from the bones where its concentration is also high. Experiments by MOTZOK (1950a) support this view. MOTZOK (1950b) has based an assay of vitamin D activity on the measurement of plasma alkaline phosphatase.

ZETTERSTRÖM (1951a,b) and ZETTERSTRÖM and LJUNGGREN (1951) have suggested that vitamin D acts by increasing the activity of alkaline phosphatases. ZETTERSTRÖM (1951a) prepared phosphorylated vitamin D_2, which is somewhat soluble in water, and thus available for enzyme studies *in vitro*. The compound was shown to increase (*in vitro*) the activities of alkaline phosphatases from bone and kidney fourfold and twofold, respectively. Phosphorylated vitamin D_2, in concentrations of 10^{-5} mol, increased the rate of oxygen uptake in a suspension of mitochondria from kidneys about twofold, more for mitochondria from rachitic rats than for such from normal rats. The

effect on the oxygen uptake was considered to be an indirect consequence of increased phosphatase activity.

Alkaline phosphatases may be important in the absorption and excretion of phosphate as well as in the deposition of calcium phosphate in the cartilage. However, the relation of the above-mentioned observations with phosphorylated vitamin D to the function of the vitamin *in vivo* still awaits confirmation. According to RÄIHÄ and FORSANDER (1952) vitamin D activates the phosphorylation of thiamin. NICOLAYSEN (1943) has suggested that in growing rats calcium absorption is regulated by an endogenous factor which is effective only in the presence of vitamin D. HENRY and KON (1953) have criticized this hypothesis.

Very interesting is the fact that if a certain amount of citrate is added to an otherwise rachitogenic diet, it is difficult to produce rickets in rats (HAMILTON and DEWAR, 1937; SHOHL, 1937). A curative effect of citrate against rickets in infants has also been claimed (SHOHL and BUTLER, 1939; GLANZMANN, MEIER, and WALTHARD, 1946). Citrate has been reported to be low (1·5 mg per cent) in serum of rachitic infants, and to rise to normal values (2·5 mg per cent) after vitamin D treatment (HARRISON and HARRISON, 1952). The role of citrate in the prevention or healing of rickets is not clear though it seems likely that the formation of soluble complexes with calcium is of importance. The influence of citrate shows that the requirement of vitamin D depends on the composition of the diet just as is the case for certain other vitamins, for instance, vitamin E. Vitamin D acts against the rachitogenic (or osteoporotic) effect of dietary calcium sulphate (GLANZMANN, MEIER, and UEHLINGER, 1946). It also markedly increases the utilization of phytin phosphorus (KRIEGER, BUNKFELDT, and STEENBOCK, 1940; KRIEGER, BUNKFELDT, THOMPSON, and STEENBOCK, 1941; BOUTWELL, GEYER, HALVERSON, and HART, 1946; SINGSEN and MITCHELL, 1945). However, vitamin D does not increase phytase activity (SPITZER, MARYUAMA, MICHAUD, and PHILLIPS, 1948), but it has been found to increase the extractable intestinal phytase of rats and chicks (STEENBOCK, KRIEGER, WIEST, and PILEGGI, 1953). Sufficient calcium must be present if vitamin D shall suppress the anticalcifying effect of phytate (MELLANBY, 1949). A study of the relationship of dietary phytate, Ca and vitamin D, to rickets in children in Dublin during the years 1943 to 1948, was made by JESSOP (1950).

(*g*) *Requirements and allowances*

The recommended allowance for vitamin D for humans given by the National Research Council, Board on Food and Nutrition, Washington, D.C. (1948), is 400 i.u. daily from birth up to the age of 20 years and for women during pregnancy (latter half) and lactation. For other (normal) adults over the age of 20 years the requirement is believed to be so small that no allowance needs to be given. The allowance shall secure prevention of rickets in infants, good growth and optimal calcium retention. Sufficient dietary calcium is, of course, a prerequisite. The observational data on which the recommendation is based are mentioned in the circular.

Certain clinical workers, e.g. HOUET (1946, 1949) who have studied the calcium and phosphate metabolism in infants in relation to the intake of vitamin D believe that vitamin D_3 is twice as active as vitamin D_2. HOUET estimates the daily requirement for infants at 250 to 300 i.u. D_3 or 500 to 600 i.u. D_2.

Tentative allowances for domestic animals are given by the U.S. National Research Council, Committee on Animal Nutrition (1944–49). HOGAN has reviewed the requirement of poultry. It is to be remembered that vitamin D_2 has little value for poultry. COUCH, JAMES, and SHERWOOD (1946) found that hens and pullets required less vitamin D when manganese was added to the ration. Vitamin D is dispensable for many domestic animals when the feed contains a favourable ratio Ca : P. Recent experiments by EWER (1951) on sheep receiving a rachitogenic diet low in P and vitamin D have shown that rickets could be prevented either by one large dose of vitamin D_2 (25 mgm) or by raising the P intake (to 1·9 gm per day). The treatment with one large dose of D_2 had no effect upon growth, whereas the increased supply of P had. The effect of various levels of Ca, P, and vitamin D intake on bone growth in foxes has been studied by HARRIS, BASSETT, and WILKE (1951).

(*h*) *Hypervitaminosis D*

It has been general knowledge for a long time that large doses of vitamin D, especially when repeated, may produce a toxic syndrome involving deposition of calcium phosphate in various organs, e.g. the kidneys. The toxic dose is rather high, about 1000 times the therapeutic dose. Some authors (working with vitamin D_2) have warned against the treatment of rickets or tetany with one very large dose (DEBRÉ, THIEFFRY, BRISSAUD, and TRELLU, 1946). In dogs, atrophy of the parathyroids has been found as part of the hypervitaminosis D syndrome. Hypervitaminosis D in a poikilotherm animal, viz. the larval frog, has been studied recently by SCHLUMBERGER (1953).

The vitamin D balance and distribution in rats given a massive dose of ergocalciferol have been investigated by E. M. CRUICKSHANK and KODICEK (1953) and ARMITAGE (1953). Forty thousand i.u. were given to weanling rachitic rats, and various organs, faeces and urine were assayed on the 4 subsequent days. About 23 per cent of the ingested dose was accounted for, the main site of deposition being the liver. A not inconsiderable part was excreted in the faeces, none in the urine. The fate of the remainder of the ingested dose was believed to be conversion into products having no antirachitic activity.

(*i*) *Substances with inhibiting effect*

A factor in hay inhibiting the action of vitamin D has been reported by WEITS (1952). BRION, FERRANDO, CHENAVIER, and PORTAL (1951) have suggested that cod liver oil contains a substance which inhibits the action of the amount of vitamin D_3 present. BRAUSE, KON, and WHITE (1943, 1944) found that high amounts of dried yeast in the ration of pigs produced rachitogenic

symptoms. This observation has been discussed by HOFF-JØGENSEN (1947, 1948) and DE MAN (1948).

(*j*) *Miscellaneous observations*

GREEP and FISCHER (1950) found that vitamin D is not essential for growth of intact rats given an adequate Ca : P intake and may impair growth in mineral imbalances. Given to parathyroidectomized rats with normal mineral intake, vitamin D improved growth greatly. PINCUS and GITTLEMAN (1952) could not find any regulatory effect of vitamin D upon the serum calcium and phosphorus levels in infants fed formulas.

Chronic dosage with dihydrotachysterol which increases blood calcium (WERDER, 1939), for instance, in hypoparathyroidism, produces bone resorption in young rats (LATTA and TRISTAN, 1950). KODICEK (1950a,b) showed that the bacteriostatic effect of long chain fatty acids on the growth of lactobacillus casei can be reversed by vitamins D_2 and D_3. This effect was utilized in a microbial assay for pure vitamins D.

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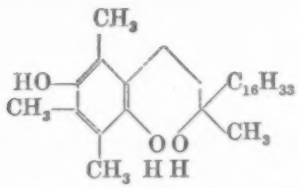
III. VITAMIN E

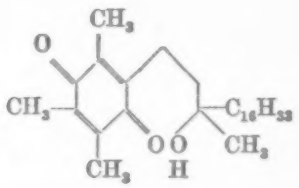
THE representatives of the vitamin E group are α-, β-, γ-, and δ-tocopherols, all of which are methyl-substituted tocols.

In the rat fertility test and in most other biological tests, α-tocopherol is much more active than the other tocopherols. The opposite applies to the *in vitro* antioxidant effect.

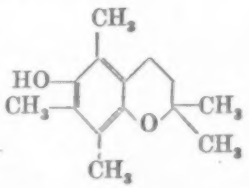
Certain substances closely related to the tocopherols are of interest to the mode of action of vitamin E, e.g. tocopherylquinone, —hydroquinone, simpler substituted oxychromanes and the free semiquinone radical (MICHAELIS and WOLLMAN, 1949):



α-tocopherylhydroquinone



α-tocopherylquinone



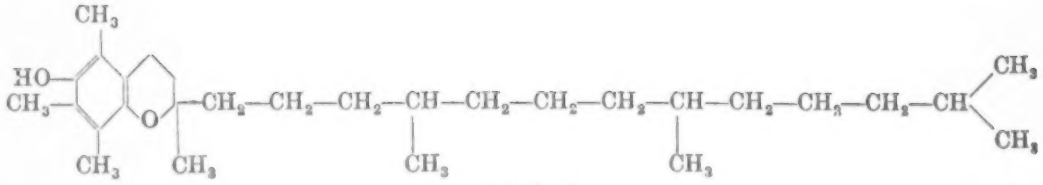
2,2,5,7,8-pentamethyl-6-oxychromane

Further, a reversible oxidation product of α-tocopherol $C_{29}H_{50}O_3$ (BOYER, RABINOVITZ, and LIEBE, 1949).

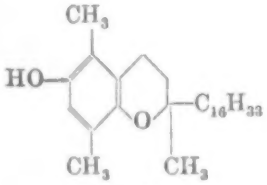
Chemical methods for determination of tocopherols in common use at present are predominantly those of QUAIFE and HARRIS (1948), QUAIFE (1948), for serum tocopherols those by QUAIFE, SCRIMSHAW, and LOWRY (1949).

The international unit of vitamin E activity measured in the rat fertility test is 1 mgm synthetic d,l-α-tocopherol acetate dissolved in 100 mgm olive oil. It is not common, however, to express vitamin E activity in units.

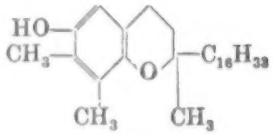
Since α-tocopherol is available both in natural d-form and as synthetic d,l-form (the point of asymmetry being the attachment of the side chain to the chromane nucleus) it would be of interest to know whether the d- and the d,l-forms are equally active biologically. M. L. QUAIFE (1952) has determined the deposition in liver, body fat, skeletal muscle, and blood serum of rats after



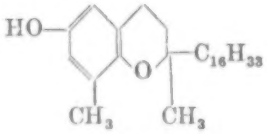
α-tocopherol



β-tocopherol



γ-tocopherol



δ-tocopherol

feeding the two forms of α-tocopherol, free as well as acetates. According to QUAIFE, the storage of tocopherol is about twice as great for the d-form as for the d,l-form. The deposition of tocopherol in the tissues decreases in the order mentioned above.

γ-Tocopherol is much less efficient in causing storage of tocopherol as α. This is in agreement with earlier studies of BARNES, LUNDBERG, HANSON, and BURR (1943) and LUNDBERG, BARNES, CLAUSEN, LARSEN, and BURR (1947) regarding the storage in depot fat of rats.

(*a*) *Deficiency symptoms*

The numerous symptoms of vitamin E deficiency may at present be classified in several ways, viz:

1. According to the tissue affected.

2. According to whether or how they are affected by certain changes of the basal diet, especially:

(*a*) According to whether they depend upon the presence of easily autoxidizable fatty acids in the diet.

(*b*) According to whether they can be counteracted by increase of dietary sulphur amino acids.

(*c*) According to whether they can be more or less markedly counteracted by artificial redox substances such as methylene blue, or by other antioxidants.

(*d*) According to whether they can be counteracted by tocopheryl quinone or tocopheryl hydroquinone, simpler substituted oxychromanes and other substances relatively closely related to vitamin E.

(*e*) According to how they are influenced by increase of several dietary components not belonging to the groups mentioned above, such as cholesterol, inositol, Mn, factor 3, etc.

3. If all vitamin E deficiency symptoms are not manifestations of the lack of antioxidant effects, one might simply divide them into symptoms which are probably due to the lack of such effects and those which are not.

It is evident that some of the above-mentioned ways of classification overlap each other and suffer from lack of sufficient data to permit a clear cut presentation. Table 1 shows in condensed form how far a classification according to 2 is applicable to a series of vitamin E deficiency symptoms on the basis of the data known to the reviewer.

It must be borne in mind that in many cases the effects of the various substances compiled from the literature have not been compared on an equimolar basis or with the same basal diet.

(*b*) *Brief discussion of symptoms with reference to Table* 1

The "classical" symptom of vitamin E deficiency: *reproduction failure in female rats* is not prevented by the omission of easily autoxidizable fat from the

Table 1. Influence of dietary changes on certain vitamin E deficiency symptoms.

Symptom	Easily autoxidizable fat		Improvement is obtained by addition of*																							
	necessary for occurrence of symptom	aggrevates or accelerates condition	Cystine		MB†		NDGA‡		Antabuse		Vit. C		Tocopheryl quinone		Tocopheryl hydroquinone		Pentamethyl oxychromane		Manganese		Cholesterol		Factor 3		Inositol	
Reproduction failure, female rats	0				+	+ NB								0		0		0								
Testicular degeneration, rats						0																				
Muscular degeneration: chicks	0	+	+		0		0		0		0														•	
calves		+				+						0														
young rats																										
adult rats	0																									
rabbits	0	+												+		+		+								
guinea pigs																										
Creatinuria (rabbits)														+		+		+								
Increased oxygen uptake of muscles (hamsters)																		+								
Degeneration of nervous tissue, rats																										
Encephalomalacia, chicks	–			0		+		(+)		0		(+)						0				+				(+)
Exudative diathesis, chicks	–			0		+		(+)		+		(+)		0								0				(+)
Brown adipose tissue, chicks, rats	+			0		+		+		+		(+)		0						0		0			•	(+)
Peroxidation of depot fat, chicks, rats				0		+		+		+		(+)		0						0		0	•			

Brown uterus rats	?					+								+																
Incisor depigmentation, rats	+			0		+		+		+		0									+								
Haemolysis with dialuric acid, rats	0	+				+ NB																								
Hepatic necrosis, rats	+ NB	+		+		+ NB						0																+		
Lung haemorrhage, rats	+ NB	+																												
Low vitamin A in liver, rats, chicks	+			0		+		(+)		+		0																		
Tubular degeneration, rats						+																								
Necrotinizing arteritis, dogs with kidney damage	+																													

* Left half of each column refers to diets without easily autoxidizable fat; right column to diets with such fat.
† MB = methylene blue.
‡ NDGA = nordihydroguaiaretic acid.

NB means that at present there is disagreement between some investigators.

diet (DAM, GRANADOS, and PRANGE, 1949), and therefore, is not likely to be due to the formation of abnormal oxidation products of these substances. Since, however, the reproduction capacity of female rats is reduced merely by exclusion of fat from the diet (EVANS, LEPKOVSKY, and MURPHY, 1934), the possibility that vitamin E is concerned with the protection of essential fatty acids in the embryo might still be a point of interest. The possible role of sulphur amino acids in relation to this symptom seems not to be definitely clarified. The protective action of continuous feeding of methylene blue against this symptom has been reported from two laboratories (DAM and GRANADOS, 1952; MARKEES, 1953), whereas MOORE, SHARMAN, and WARD (1953a) failed to confirm it. According to MARKEES, methylene blue does not act when given to vitamin E depleted rats several times during pregnancy. This could signify that continuous feeding of methylene blue may act by protecting vitamin E, thereby delaying the depletion. In the experiments of DAM and GRANADOS (1952) and CHRISTENSEN and DAM (unpublished) MB was effective also when the diet contained no added fat.

Tocopheryl quinone (GOLUMBIC and MATTILL, 1940) and tocopheryl hydroquinone (ISSIDORIDES and MATTILL, 1951) as well as penta methyloxychromane (MARKEES, 1953) have been found inactive against this symptom.

Muscular degeneration originally studied with rabbits, guinea pigs, hamsters, young and adult rats, and with ducklings is very easy to develop in chicks (DAM, PRANGE, and SØNDERGAARD, 1952c). This symptom appeared even when the basal diet did not contain fat; methylene blue, vitamin C, NDGA and antabuse did not prevent it. However, the symptom is markedly counteracted by increase of dietary cystine. It is possible, therefore, that this symptom is connected with some abnormal metabolism of sulphur amino acids. BLAXTER, BROWN, and MACDONALD (1953) have recently continued their studies on muscular dystrophy in calves (BLAXTER and WOOD, 1952; BLAXTER, WATTS, and WOOD, 1952; MACDONALD, BLAXTER, WATTS, and WOOD, 1952). With diets containing a small amount of α-tocopheryl acetate addition of cod liver oil or certain fractions thereof caused dystrophy. These fractions were those containing poly-unsaturated fatty acids. The authors conclude that the toxicity of cod liver oil to calves is due to its content of poly-unsaturated fatty acids, and not to any hypervitaminosis A or D; they suggest that the toxicity is a general effect of poly-unsaturation rather than of a single poly-ethenoid acid. Very important is the finding that the dystrophic muscles of calves did not contain less total tocopherols than normal muscles.

In a further study (BLAXTER, BROWN, WOOD, and MACDONALD, 1953) on muscular dystrophy in calves caused by diets containing cod liver oil (and a small amount of α-tocopherol) these authors found a protective effect of methylene blue against the toxicity of cod liver oil. This effect was not associated with higher concentrations of tocopherols in serum, muscle, liver or perinephric fat. When α-tocopheryl acetate was given by mouth, serum tocopherol increased and protection was obtained. Ascorbic acid, ethyl gallate and biotin did not protect against muscular dystrophy caused by cod liver oil.

In connection with the effect of cod liver oil in producing changes which can be corrected by vitamin E it is worthwhile to remember that cod and other fish liver oils contain some vitamin E. The most recent figures for α-tocopherol in fish oils are presumably those found by BROWN (1953) varying from 18 to 45 mgm α-tocopherol per 100 gm.

Creatinuria and muscular degeneration were found (in rabbits) by MACKENZIE, ROSENKRANTZ, ULICK, and MILHORAT (1950) to be counteracted by tocopheryl quinone and the corresponding hydroquinone. The author discussed the activity of these substances in relation to a participation of them and vitamin E in an oxidation reduction system in the body. The same symptom as well as the accompanying *increase of oxygen uptake* in muscle homogenates have also been found to be counteracted by penta methyl-6-oxy-chromane (MARKEES, 1953).

Recently, FARBER, MILMAN, and MILHORAT (1953) have studied the effectiveness of several other substances related to tocopherol against muscular dystrophy in rabbits. They found that tocopheramin, α-tocopheroxide and mercapto-tocopherol acetate are active against muscular dystrophy when given in sufficient doses. These substances are also active in the usual fertility test. Trimethyl-phytyl-benzoquinone and trimethyldihydrophytyl-benzoquinone given in high doses were active in preventing dystrophy in spite of their ineffectiveness in preventing sterility in female rats. The authors emphasize the difference between vitamin E activity measured by the effectiveness against muscular dystrophy in rabbits and impaired reproduction capacity in female rats.

MACKENZIE and MACKENZIE (1953) suggest that the hydroquinone is specifically active against muscular dystrophy: α-tocopherol may be converted into the hydroquinone *in vivo*, thus functioning as a provitamin for the latter, whereas the hydroquinone is not converted into α-tocopherol which prevents sterility.

Degeneration of nervous tissue associated with paralysis in adult rats (RINGSTED, 1935; EINARSON and RINGSTED, 1938) has been re-investigated by EINARSON (1953). Unfortunately, his demonstration of the acid fast staining fluorescent substance previously found by other investigators (e.g. WOLF and PAPPENHEIMER, 1945) in nervous and other tissue of vitamin E deficient animals receiving easily autoxidizable fatty acids in their diet, was not studied in relation to the components of the diet. EINARSON is of the opinion that the muscular degeneration seen in adult rats is a consequence of the impairment of the nervous system; he argues against other authors, e.g. TELFORD (1941), WOLF and PAPPENHEIMER (1942), who believe that the muscular degeneration is primary. The development of neuromuscular damage in rats on vitamin E deficient, fat-free diets, was shown by MACKENZIE, MACKENZIE, and MCCOLLUM (1939).

Encephalomalacia and *exudative diathesis* in chicks as well as *brown adipose tissue* and *peroxidation of the depot fat* in chicks and rats all depend upon the presence of easily autoxidizable fatty acids in the diet. The same applies to

incisor depigmentation in rats. These symptoms are uninfluenced by cystine but to varying degree ameliorated by NDGA and methylene blue. Vitamin C (0·3 per cent) gives fairly good protection against exudative diathesis and some protection against encephalomalacia, brown adipose tissue and peroxidation of depot fat. Several of these symptoms are also counteracted by such antioxidants as antabuse and by sulphaguanidin (DAM, KRUSE, PRANGE, and SØNDERGAARD, 1948, 1951; GRANADOS, AAES-JØRGENSEN, and DAM, 1950; AAES-JØRGENSEN, DAM, and GRANADOS, 1951). It seems justified, therefore, to assume that these symptoms are caused in some way by the autoxidation *in vivo* of ingested easily autoxidizable fat in the absence of vitamin E as antioxidant.

The *exudative diathesis* does not occur when fat is not present in the diet (DAM, 1943, 1944). (An oedematous condition of a different type may occur in chicks reared on fat-free diets, but this condition is unrelated to vitamin E deficiency (REISER, 1950: DAM, PRANGE, and SØNDERGAARD, 1952a,c). The oedemas reported by BIRD and CULTON (1940) may be of this nature but aggravated by the ingestion of small amounts of cod liver oil.) Both cod liver oil and lard may provoke the exudative diathesis. The effect is connected with the highly unsaturated fatty acid fraction. The saturated acids, oleic acid, and the non-saponifiable fraction are inactive. The total fatty acid fraction from linseed oil may produce exudative diathesis in chicks, but cod liver oil is the most active among the fats mentioned, and when it is used a yellow-brown colour is seen in the affected adipose tissue after the exudate has been reabsorbed. If the highly unsaturated fatty acids are autoxidized (*in vitro*) to such an extent that the iodine value decreases sufficiently, the fat will not cause the disease when ingested.

The circumstance that the disease primarily affects adipose tissue facilitates an examination of the affected tissue for abnormal oxidation products of fatty acids, for instance, lipoperoxides. DAM and GRANADOS (1945a) undertook such an examination using the iodometric method of KING, ROSCHEN, and IRVIN (1933), slightly modified for the purpose. The diet contained 5 per cent cod liver oil.

It was found that the symptom began approximately at the time when the presence of peroxides could be detected whereas higher peroxide values were found in the later stages of the disease, viz. when the yellow-brown colour of the fat appeared. In chicks receiving vitamin E or no fat the adipose tissue did not contain peroxides. DAM, PRANGE, and SØNDERGAARD (1952a) found peroxides in the adipose tissue of chicks, reared on vitamin E deficient diets containing 10 per cent lard and no cod liver oil.

The question then arises whether the formation of abnormal oxidation products of fat is the cause of the exudative diathesis or merely a sign of the disappearance of vitamin E from the tissue. (It must be borne in mind that the initiation of autoxidation of fat in the adipose tissue will also hasten the disappearance of vitamin E.) As the matter stands at present, the author considers the first alternative to be likely because the disease has not been

observed in the absence of dietary highly unsaturated fatty acids, even when the diet contains a substance such as 2 : 6-dichlorophenolindophenol which oxidizes free tocopherols. The explanation may be that some primary autoxidation product of the fatty acids (not necessarily the peroxides, but perhaps more likely a free radical) reacts with substances in the wall of the capillaries, and thereby damages the latter. Methylene blue which counteracts the onset of exudates may act directly as antioxidant, but it also delays the depletion of vitamin E in the fat tissue (DAM, KRUSE, PRANGE, and SØNDERGAARD, 1951).

Encephalomalacia is favoured and exudative diathesis depressed by a diet containing high lard and high protein. The search for peroxides in the lipids of brains from chicks with encephalomalacia has, hitherto, been unsuccessful. (This does not mean, however, that autoxidation has not occurred, since it is known that certain difficulties are involved in the determination of peroxides in phosphatides, which constitute a large part of brain lipids.) Diets which produce encephalomalacia in chicks will cause muscular dystrophy in ducklings (PAPPENHEIMER and GOETTSCH, 1934) and patchy necrosis of the gizzard in turkey poults (JUNGHERR and PAPPENHEIMER, 1937).

The yellow-brown fat in the adipose tissue shows a yellow-green fluorescence under the ultraviolet lamp. It has been demonstrated (DAM and GRANADOS, 1945a) that the development of yellow-brown fat is associated with peroxidation of the fat *in vivo*. The fatty acids from cod liver oil favour the development of the brown fat much more than those from lard (GRANADOS and DAM, 1945a). The yellow-brown colour of the fat is due to the presence of two brown components, one of which is fat-soluble while the other is insoluble in fat solvents as well as in water. The non-fat-soluble component stains acid-fast with fuchsin and resembles thereby the pigment "ceroid" (LILLIE, DAFT, and SEBRELL, 1941; GYÖRGY and GOLDBLATT, 1942; LILLIE, ASHBURN, SEBRELL, DAFT, and LOWRY, 1942) which occurs in liver, lymph nodes and other tissue of rats reared on diets causing liver damage and containing cod liver oil. There is, in fact, no definite distinction between "ceroid" and the non-fat-soluble component of the brown adipose tissue.

A method for the determination of fat-peroxides based on the oxidation of colourless leuco-dichloro-phenol-indophenol to the coloured compound was developed by HARTMANN and GLAVIND (1949), and adopted for use on frozen sections of adipose tissue (GLAVIND, GRANADOS, HARTMANN, and DAM, 1949). By this method it was found that the initial stage in the development of the pigments is characterized by a homogenous peroxidation of the fat content of some of the cells, but the fat-insoluble component which appears as granules does not necessarily contain peroxides (GRANADOS and DAM, 1945b).

The pigments in the yellow-brown fat tissue must have originated from the abnormal oxidation of the highly unsaturated fatty acids occurring in the adipose tissue. The fat-soluble component must represent the least transformed oxidation products while the non-fat-soluble component consists of polymerization products beyond the peroxide stage. Cod liver oil autoxidized *in vitro* will

also give rise to yellow-green fluorescent fat-soluble and non-fat-soluble reaction products, the latter staining acid-fast with fuchsin.

The reason why, in rats, the haemorrhagic and exudative stages do not occur —contrary to what is the case in chicks—could be sought in differences in the fine structure of the capillaries in the two species. Further histochemical studies must decide whether this explanation is correct.

Fasting does not increase the tendency to autoxidation of body fat in rats. Highly unsaturated fatty acid as well as tocopherol are diminished much less than total fat (CHEVALLIER, BURG, SCHNEIDER, and WAGNER, 1950).

The depigmentation of the incisors of rats was first described by DAVIES and MOORE (1941). The fact that fat is necessary for the appearance of the symptom was demonstrated by GRANADOS and DAM (1945b). The normal brown pigment of the incisors is a ferric compound, probably ferric phosphate. The maxillary incisors, which are normally more coloured than the mandibular, are the first to lose their colour when the rats are placed on a vitamin E deficient diet containing suitable fat. Not only cod liver oil but also lard will cause depigmentation of the incisors. Contrary to this, lard is much less effective in causing brown colouration of the adipose tissue (if it does so at all). It is the highly unsaturated fraction of the fatty acids which cause the depigmentation of the incisors in vitamin E deficiency (DAM and GRANADOS, 1945b). The iron content of the enamel decreases very much and manganese rises somewhat under the influence of the "depigmenting" diet (DAM, GRANADOS, and MALTESEN, 1950). The effect is probably in some way related to the lack of an antioxidant effect against autoxidation of fatty acids in the enamel organ. The beneficial effect of methylene blue against incisor depigmentation in rats was not verified by MOORE (personal communication).

The brown colouration of the uterus in female rats originally found by MARTIN and MOORE (1936) was seen by DAM and GRANADOS (1945a) also in rats receiving no dietary fat, although it was less marked than when cod liver oil was given. It would, *a priori*, seem likely that this symptom is due to the same substances as those responsible for the brown adipose tissue; in that case, however, the uterus should be able to retain its content of highly unsaturated fatty acids long enough to enable the autoxidation products to accumulate during the period of vitamin E deficiency even if no fat was given. This point has not been sufficiently elucidated yet, but is in agreement with the findings of HOLMAN and GREENBERG (1953) according to which reproductive tissue lipids have a particularly high content of polyunsaturated acids. MOORE and WANG (1937) suggested that oxidized protein is contained in the brown pigment. MOORE, SHARMAN, and WARD found (with diets containing cod liver oil and lard) that the brown colouration of the uterus could be prevented by methylene blue (1953a), thiodiphenylamine, Bindschedler's green (1953b) malachite green and "new methylene blue" (1953c), the effect of these substances must be related to their redox properties, probably in connection with an antioxidant action. MARKEES (personal communication) found protective effects with tocopherylquinone.

Very interesting is the demonstration by GYÖRGY and ROSE (1948) and ROSE and GYÖRGY (1950) that *alloxane and dialuric acid produce haemolysis* in rats reared without vitamin E. The symptom in alloxan poisoned rats was originally found to be aggravated when the diet contained lard instead of no fat. The haemolysis test may be carried out *in vitro*. ROSE and GYÖRGY (1952) found that hydrogen peroxide may be used instead of dialuric acid to effect the haemolysis *in vitro*. The effect of both of these agents is counteracted by adding α-tocopherol or catalase *in vitro*. Also certain antioxidants such as the other tocopherols or substituted hydroquinones are active when added *in vitro*. The symptom of easy haemolysis with dialuric acid or hydrogen peroxide must be due to lack of an antioxidant effect. Continuous feeding of methylene blue was found in the reviewer's laboratory, but not in MOORE's, to minimize the haemolysis (CHRISTENSEN and DAM, 1951; HEARD, MOORE, and SHARMAN, 1952). ROSE and GYÖRGY (1952) reported that when administered *in vivo* the effectiveness of the tocopherols in inhibiting dialuric acid haemolysis *in vitro* decreased in the order α, β, γ, δ, similarly to their activity measured by foetal resorption.

The circumstance that the antioxidant activity of the tocopherols *in vitro* increases from α to δ, whereas the biological activity as measured in the fertility test with female rats decreases in the same order is no hindrance for the assumption of an antioxidant effect *in vivo*. BARNES, LUNDBERG, HANSON, and BURR (1943) and LUNDBERG, BARNES, CLAUSEN, LARSEN, and BURR (1947) have shown that the biopotencies of the various tocopherols as antioxidants (measured in the rendered fat of the experimental animals (rats)) is related to the amounts deposited in the fat, α being deposited to a larger extent than γ. DAM and GRANADOS (1945) found that when fed (as acetates) in the same relatively small doses α-tocopherol prevented the exudative diathesis and peroxidation of body fat in chicks whereas γ-tocopherol did not.

Hepatic necrosis (SCHWARZ, 1944; GYÖRGY, 1947) and *fatal lung haemorrhage* (HOVE, COPELAND, and SALMON, 1949) occur in vitamin E deficiency under certain dietary conditions, partly in relation to the amount and quality of the protein.

Fat with a high content of highly unsaturated fatty acids seems necessary for the production of the two symptoms in rats within 10 weeks (DAM and GRANADOS, 1952) or of liver necrosis within 172 days (MCLEAN and BEVERIDGE, 1952). The latter authors are inclined to expect that liver necrosis might have developed on fat-free diets if the feeding period had lasted longer. SCHWARZ (personal communication) has developed liver necrosis in rats on fat-free diets.

When the dietary protein consists of "damaged" casein (SCHWARZ, 1944) or is provided by certain types of yeast (GYÖRGY, ROSE, TOMARELLI, and GOLDBLATT, 1950) the incidence of hepatic necrosis is particularly high.

Low sulphur amino acids, especially low cystine, is one of the conditions for the development of hepatic necrosis (HOCK and FINK, 1943; GLYNN and HIMSWORTH, 1944; GYÖRGY and GOLDBLATT, 1949; SCHWARZ, 1952b).

A more detailed review of this subject has been given by HIMSWORTH (1950) and MCLEAN and BEVERIDGE (1952). However, the dietary conditions are still

more complicated, since SCHWARZ (1951, 1952a) has demonstrated the existence of a "factor 3" in non-necrogenic yeast and in casein which protects against the symptom. The factor is not identical with cystine or methionine, which, under suitable conditions will protect against liver necrosis in vitamin E deficiency. LINDAN and WORK (1951, 1953) have shown that both reduced and total glutathione as well as ascorbic acid are very much lowered in the necrotic livers of vitamin E deficient rats. While cystine protects, ascorbic acid does not. The liver necrosis-lung haemorrhage syndrome also occurs in chicks (DAM, PRANGE, SØNDERGAARD, 1952c and unpublished data).

It might be that the effect of vitamin E in preventing liver necrosis consists in the protection of cystine, glutathione and some other sulphur containing metabolites essential to the normal state of the liver. The destruction of these metabolites may be accelerated by autoxidizable fat.

Recently, LINDAN and WORK (1953) have found the same changes in reduced glutathione, ascorbic acid, non-protein and total nitrogen and glycogen in liver lobes rendered necrotic by circulatory congestion as previously found in dietary necrosis. They suggest that these chemical changes could be the result of changes occurring in the dead or dying liver, and not necessarily be specific consequences of the dietetic lesion. However, since cystine protects, it is likely that the protection of sulphur compounds by vitamin E is essential to the prevention of the symptom.

In connection with the role of vitamin E in maintaining the normal state of the liver, the protective effect against damage of this organ by carbon tetrachloride and pyridine is of interest (HOVE, 1948, 1953a).

HOVE (1953a) found methylene blue, and to a lesser degree, yeast nucleic acid, to provide protection against pyridine poisoning. Pyridine was found to catalyze the oxidation of lard and other fats and to depress the antioxidant effect of α-tocopherol.

HOVE and HARDIN (1951a,b) found that the improvement obtained by an 18 per cent casein level instead of a 10 per cent level, in vitamin E free diets for CCl_4 poisoned rats, could be masked by adding both cystine and vitamin B_{12} to the low casein diet. In the absence of these factors the vitamin E requirement is increased and creatine formation was dependent on vitamin E.

HOVE (1953b) found that the toxic symptoms caused by tri-o-cresyl-phosphate reported by other authors to resemble vitamin E deficiency was more efficiently counteracted by high dietary casein than by vitamin E. The substance acted as a pro-oxidant.

MOORE (1940) found that the feeding of vitamin A to rats (1,000 i.u. A per week in the form of halibut liver oil on a diet containing 10 per cent lard) resulted in higher *vitamin A levels in the liver* when the diet contains vitamin E than when the diet is vitamin E-free. This effect has been confirmed in several laboratories. HICKMAN, KALEY, and HARRIS (1944) found α-, β-, and γ-tocopherols equally active as regards the vitamin A sparing effect. They also found that the utilization of vitamin A is more efficiently increased by free tocopherol than by its esters. This observation was also made by MILES, ERICKSON, and

MATTILL (1949) who further found that increase of the vitamin E content beyond a certain level results in suboptimal utilization of vitamin A.

DAM, PRANGE, and SØNDERGAARD (1952a,b) found no significant effect of vitamin E on vitamin A storage in the liver of chicks within 5 weeks and rats within 14 weeks when the diet contained no cod liver oil (vitamin A being given as natural ester), whereas the effect was marked on diets containing cod liver oil. They also found that the vitamin A level in the liver of rats decreased from the 5th to the 14th week when the cod liver oil containing diet was E-free, and that methylene blue, thiodiphenylamine, and antabuse, but not cystine and vitamin C, increased vitamin A storage to a very high degree.

All these observations are in agreement with the assumption of an *in vivo* destruction of vitamin A coupled to the autoxidation of highly unsaturated fatty acids in the absence of antioxidants.

HOVE and HARRIS (1946) have shown that vitamin E increases the utilization of a suboptimal level of essential fatty acids in the diet of rats. The explanation is apparently that vitamin E protects these metabolites against abnormal oxidation. Therefore, one may conclude that when highly unsaturated fatty acids are given in small amounts they disappear in the absence of vitamin E. When given in high amounts, under the same condition, abnormal oxidation products may accumulate or give rise to abnormal changes in the tissue.

Degeneration (cloudy swelling) of the tubuli contorti in the rat kidney was found by MARTIN and MOORE (1939). MOORE, SHARMAN, and WARD (1953a) found (with diets containing easily autoxidizable fat) that methylene blue prevents this symptom. Lack of essential fatty acids has been reported to give rise to a similar symptom (BORLAND and JACKSON, 1931; MOORE, 1937).

Necrotizing arteritis in dogs produced by a combination of high fat diet and experimental renal insufficiency (RUSSELL T. HOLMAN, 1950) is reported to be dependent upon vitamin E deficiency and presence of unsaturated fatty acids apparently accumulating in a certain region of the convoluted tubuli.

TAPPEL (1953) has found that α-tocopherol inhibits haematin catalyzed oxidations.

Summing up the bulk of information available, the following main conclusions may be drawn:

Certain vitamin E deficiency symptoms appear only when the diet furnishes a certain amount of easily autoxidizable fatty acids. These symptoms can be more or less completely prevented by antioxidants not belonging to the vitamin E group. It is therefore likely that they are caused by autoxidation products of the autoxidizable fatty acids *in vivo*, and that in this case vitamin E acts by inhibiting the *in vivo* autoxidation process.

The effect of easily autoxidizable fat in producing these symptoms must be distinguished from the effect of such fatty acids in causing a rapid disappearance of vitamin E, an effect which will, probably, accelerate or aggravate all vitamin E deficiency symptoms. In exerting its antioxidant effect in the chain reaction by which the highly unsaturated fatty acids autoxidize free tocopherol is

supposed to be converted via its free radical and other stages into irreversible oxidation products.

Other symptoms occur even when the diet does not contain easily autoxidizable fatty acids. Some symptoms may be prevented by increasing the amount of dietary sulphur amino acids. In this case there is the possibility that vitamin E acts by protecting certain sulphur metabolites against destruction.

Symptoms which do not fall within the above-mentioned groups may be manifestations of the lack of other functions of vitamin E.

(c) *Vitamin E and enzymes*

Variation of the activity of enzymes due to vitamin E deficiency has been studied by several authors, especially after HOUCHIN (1942) reported an increased succinic dehydrogenase activity in muscles of dystrophic hamsters. BASINSKI and HUMMEL (1947) failed to confirm this report and found that *in vitro* additions of tocopherol phosphate or tocopherol succinate inhibited the succinic dehydrogenase of normal and dystrophic muscle to an equal degree. AMES and RISLEY (1949) found the phosphates of α-, γ-, and δ-tocopherol equally active inhibitors of the succinic dehydrogenase system and maintain that the effect is due to precipitation of calcium. JACOBI, ROSENBLATT, WILDER, and MORGULIS (1950) also found the activity of succinic dehydrogenase unchanged in muscles from rabbits with incipient dystrophy. JACOBI, ROSENBLATT, CHAPPELL, and MORGULIS (1950) suggest that the inhibiting effect of α-tocopheryl phosphate on the succinoxidase system of rat liver and muscle is located on some unidentified mediator operating between cytochrome b and cytochrome c. This group of authors considered the possibility that α-tocopheryl phosphate might act by precipitating Ca; they therefore omitted Ca from the system. In another study JACOBI, CHAPPELL, and MORGULIS (1950) argued against the possibility that the effect of α-tocopheryl phosphate is a non-specific detergent action.

Antithromboplastic activity of α-tocopheryl phosphate has been reported by KAY and BALLA (1950). It is, however, still doubtful whether the effects of water-soluble tocopheryl esters *in vitro* have any relation to the physiological function of vitamin E.

Inhibition of hyaluronidase by tocopheryl phosphates (α more efficient than γ, γ more efficient than δ) and by α-tocoherol was reported by MILLER and DESSERT (1949). Inhibition of lipoxidase by tocopherol was discussed by HOLMAN (1947).

BARBER, BASINSKI, and MATTILL (1949) found the aspartic glumatic transaminase activity decreased in muscle homogenates from dystrophic guinea pigs and rabbits. Decreased liver esterase activity in vitamin E deficient rats was reported by KASLBEEK and VAN DER MEER (1950). Decreased activity of alkaline phosphatase in testes and adrenals of rabbits reared on vitamin E deficient diets or on natural diets plus cod liver oil was reported by CORMIER

(1948). High amounts of xanthine oxidase were found in liver homogenates from vitamin E deficient rabbits (DINNING, 1953). REICHERT and WESTERFIELD (1953) confirmed this observation and found that the increase of xanthine oxidase activity in the liver was not accompanied by an increase of molybdenum. In a series of other tissues from vitamin E deficient rabbits xanthine oxidase was not detected.

(*d*) *Vitamin E in the human*

The content of vitamin E (total tocopherol and α-tocopherol) in common foods has been determined by HARRIS, QUAIFE, and SWANSON (1950). On the basis of their results they estimate the average daily *per capita* consumption in the U.S. to be 14 mg d,l-α-tocopherol. The richest sources are certain of the vegetable oils, cereal products and eggs are next in order of importance, animal products are poor sources.

The content of tocopherol in human milk was determined by HARRIS, QUAIFE, and O'GRADY (1952). The tocopherol content was correlated with the fat content of the milk. The values were between 0·10 and 0·48 mg per 100 ml milk with an average of 0·24 mg per 100 ml or about 80 μgm per gm of fat. No loss of tocopherol occurred on pasteurizing human milk. Human colostrum contained 1·3 to 3·6 mg per 100 ml (QUAIFE, 1947).

The occurrence of symptoms in humans traceable to lack of vitamin E is still a problem. The great difficulty in providing a satisfactory control material has rendered it impossible to demonstrate vitamin E deficiency as a factor in habitual abortion. Vitamin E therapy in muscular diseases has not been successful. Claims for a beneficial effect of vitamin E in heart disease have been met with scepsis. Discussions by various authors of diseases in relation to vitamin E are found in the Annals of the New York Academy of Science (1949). A certain interest has focused around rheumatic diseases (ANT and CYAN, 1949), Dupuytren's contracture (KIRK and CHIEFFI, 1952), periferal venous disease (BURGESS and PRITCHARD, 1948; JESSEN, GLAVIND, HARTMANN, and DAM, 1951) and retrolental fibroplasia (OWENS and OWENS, 1949) without claiming vitamin E deficiency as a primary cause of these diseases.

The tocopherol level in the blood serum of pregnant women increases from about the 17th week until parturition, viz., from values of about 1 mgm per cent to 1·5 mgm per cent (SCRIMSHAW, GREER, and GOODLAND, 1949). Nevertheless, the level in the serum of the newborn is below that of normal adults (MOYER, 1950; WRIGHT, FILER, and MASON, 1951). In this respect vitamin E behaves like other fat-soluble vitamins. MASON, DJU, and FILER (1952) determined tocopherol in various tissues of premature and full term infants and in individuals 13 to 17 years of age as well as in older individuals. The values were low at birth compared to later stages of life.

GYÖRGY, COGAN, and ROSE (1952) studied the haemolysis by dilute hydrogen peroxide of erythrocytes of newborn. They found that the haemolysis could be prevented by vitamin E *in vitro* or *in vivo* (by mouth to the newborn infant). However, even with high daily doses of vitamin E (500 mgm of mixed

tocopherols) given the mother during the last weeks of pregnancy the haemolysis test in the newborn remained unaltered.

GERLÓCZY (1949) has claimed a prophylactic as well as a curative effect of vitamin E against scleroedema in premature newborn. According to this author, scleroedema has been frequently observed in Hungary in the immediate post-war period.

MINKOWSKI (1950) has suggested low vitamin E as playing a role in capillary fragility in the premature newborn. The claim that vitamin E influences blood coagulation has prompted clinical studies in patients with vascular disease (WAGNER, 1952). GEILL (personal communication) has found that the coagulation time (not the prothrombin time) determined by the method of HOWELL-GRAM (GRAM, 1921) is actually somewhat longer in older patients when α-tocopherol acetate (150 to 200 mgm per day) is administered orally.

(e) *Vitamin E in farm animals, fur animals, etc.*

Death from heart failure after calving has been reported in cows reared with low vitamin E (GULLICKSON, 1949), although such diets did not greatly diminish reproduction capacity. Goats have been carried through several generations on vitamin E low diets (UNDERBJERG, THOMAS, and CANNON, 1938).

An increase of milk production and fat content of the milk of dairy cows as a result of fortifying their ration with α-tocopherol acetate was reported by HARRIS, SWANSON, and HICKMAN (1947). GULLICKSON, FITCH, and GILMORE (1948) failed to find such an effect.

In Denmark, NIELSEN, FISKER, PEDERSEN, PRANGE, SØNDERGAARD, and DAM (1953) conducted similar studies through two winter seasons. Addition of 2 gm d,l-α-tocopherol acetate per cow per day to two typical Danish winter rations did not result in an increased yield of milk or milk fat. The keeping quality of the butter was practically uninfluenced or possibly slightly reduced by the supplementation which caused a rise in the tocopherol content of the butter from 10 to 12 μgm per gm in the unsupplemented group to 23 to 26 μgm per gm in the supplemented group. These results are in agreement with those of SWARTLING (1949) who found that direct incorporation of α-tocopherol (2·2 to 10 mgm per 100 gm) into butter reduced the keeping quality. Transfer of ingested vitamin E from cows to the calf via colostrum was demonstrated by PARRISH, WISE, LATSCHAR, and HUGHES (1950).

Symptoms due to the feeding of cod liver oil or other fat rich in highly unsaturated fatty acids have been found in several instances. FERRANDO, CHENAVIER, and CORMIER (1949) demonstrated a decrease of the tocopherol level in the blood of dairy cows and decrease of the fat content of their milk as a consequence of feeding cod liver oil. The development of dystrophy in calves fed cod liver oil and a limited amount of tocopherol was studied by BLAXTER *et al.*, as already mentioned (p. 184).

ROBINSON and COEY (1951) have found brown fat in pigs reared on a vitamin E-deficient diet containing cod liver oil. Exudative diathesis in pigs reared under similar conditions has been reported by GARTON and NAFTALIN (1953).

Yellow fat and oedema of the fat tissue have been found in mink reared on fish scrap (HARTSOUGH and GORHAM, 1950). This fat tissue stains also acid-fast with fuchsin (MASON and HARTSOUGH, 1951). Vitamin E prevents the development of this symptom in mink and linolenic acid provokes it (LALOR, LEOSCHKE, and ELVEHJEM, 1951). The disease has been studied further by DALSGAARD-MIKKELSON, KVORNING, MOMBERG-JØRGENSEN, PETERSEN, and SCHAMBYE (1953).

Encephalomalacia has been found in chicks under field conditions (PAPPENHEIMER, GOETTSCH, and JUNGHERR, 1939). Also the exudative diathesis has recently been reported to have occurred in chicken farms in New Zealand (THOMPSON and SMITH, 1953).

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IV. VITAMIN K

THE group of vitamins K comprises the two naturally occurring representatives vitamins K_1 and K_2 and a series of simpler compounds which may act as vitamin K substitutes, especially in simple vitamin K deficiency.

The number of such compounds, particularly those with low activity is very large. They are reviewed for instance in:

H. R. ROSENBERG: *Chemistry and Physiology of the Vitamins*, Interscience Publishers, 1942.

HANS VOGEL: *Chemie und Technik der Vitamine*, 3. Aufl. 1. Bd., bearbeitet von H. KNOBLOCH. Ferdinand Enke Verlag, Stuttgart, 1950.

H. J. DEUEL, Jr., *The Lipids, Their Chemistry and Biochemistry*, Vol. I, Interscience Publishers, 1951.

The reviews also deal with the general chemistry and the synthesis of these compounds, as well as of the natural K vitamins.

The vitamin K active compounds listed below are limited to vitamins K_1 and K_2 menadione and certain therapeutically important substances. Phthiocol,

which is relatively weak, is mentioned because of its historic interest as the first known instance of a naphthoquinone derivative with vitamin K activity. Further, those substances which are sometimes mentioned in the literature as "K" with a subscript are listed. This way of designating artificial vitamin K substitutes is not very practical, however, because of the large number of compounds entitled to designation in similar way.

A systematic study of synthetic substances of the vitamin K_1 and K_2 types, but with varying length of the side chains, has been made recently by ISLER *et al.* (1953). The total synthesis of vitamin K_1 (with synthetic Isophytol) was published by ISLER (1954). Biologically it did not differ from natural vitamin K_1.

Vitamin K_1, 2-methyl-3-phytyl-1,4-naphthoquinone, Phylloquinone.

Vitamin K_2, 2-methyl-3-difarnesyl-1,4-naphthoquinone.

2-methyl-1,4-naphthoquinone, Menadione, Menaphthone, "K_3".

2-methyl-1,4-naphthohydroquinone diacetate, "K_4".

2-methyl-4-amino-1-naphthol hydrochloride "K_5".

2-methyl-1,4-diamino-naphthalene dihydrochloride, "K_6".

2-methyl-4-amino-1-naphthol hydrochloride "K_7".

Vitamin K_1 oxide.

Menadione sodium bisulphite.

Tetrasodium or dicalcium salt of 2-methyl-1,4-naphthohydroquinone (Synkayvite, Synkavit).

2-methyl-3-hydroxy-1,4-naphthoquinone, Phthiocol.

Besides the substances mentioned, also α-naphthol with a methyl group in the 2 or 3 position is relatively active as vitamin K. The same applies to the corresponding methyltetralones.

The indications of potency of the various more or less vitamin K active compounds found in the literature refer mostly to determinations carried out in the early days of vitamin K research and are not very accurate.

The potencies per mol of vitamin K_1, K_2, menadione and the easily hydrolyzed esters of its hydroquinones seem to be of the same order of magnitude when tested against simple alimentary vitamin K deficiency in chicks, allowing a sufficient time for the action to develop. When measured ½ to 1 hr after the intravenous administration, vitamin K_1 is more active than menadione (DAM and SØNDERGAARD, 1953).

Biological assay: The method of determining vitamin K activity used in the reviewer's laboratory (DAM, KRUSE, and SØNDERGAARD, 1951) is based on the determination of the prothrombin time in blood from the carotid artery of chicks before and 20 hr after the administration of one single dose of the substance to be tested, menadione being used as standard substance. Because of the different velocity of action found for vitamin K_1 and menadione, it may not be strictly correct to use as standard another form of vitamin K than the one contained in the material to be tested, at least not without indication of the assay method used. Considerations of this nature have been mentioned by ALMQUIST (1952).

(*a*) *Occurrence*

As commonly known, vitamin K_1 occurs in green leaves associated with chlorophyll in the chloroplasts. For this reason the designation Phylloquinone was introduced by KARRER on the reviewer's suggestion (KARRER and GEIGER, 1939).

Vitamin K_2 occurs in putrified material, where it is formed in bacteria. A pure culture of the spore-forming soil bacteria, *Bacillus brevis*, has been shown to contain this form of vitamin K (TISHLER and SAMPSON, 1948).

Phthiocol was originally isolated from Mycobacterium tuberculosis by ANDERSON and NEWMAN (1933), by a procedure involving alkali treatment. FRANCIS, MADINAVEITIA, MACTURK, and SNOW (1949) have isolated from the same bacteria a substance resembling vitamin K_2 but apparently containing a longer side chain. They found that on alkaline hydrolysis this substance yields phthiocol. It is therefore possible that phthiocol is not present as such in Mycobact. tuberculosis.

(*b*) *Determination of K-vitamins by chemical and physical methods*

These rather numerous methods have been reviewed by the author up to the year 1948 (DAM, 1948). The most convenient chemical method for vitamin K_1 is colorimetry of the blue colour developed with sodium diethyldithiocarbamate in alkaline alcoholic solution (IRREVERRE and SULLIVAN, 1941). However, the extraction from biological material and purification of such extracts before the colour reaction involve considerable difficulty.

Menadione is relatively easy to determine in pharmaceutical preparations, for instance by the method of SCUDI and BUHS (1941), based on the development of a yellow colour with cysteine and sodium hydroxide, or the reaction with dinitrophenylhydrazine by NOVELLI (1941), modified by MENOTTI (1943).

(*c*) *Mode of action*

In the early days of vitamin K research, the conception of blood coagulation was still the classical Schmidt-Morawitz-Arthus-Hammarsten theory according to which thromboplastin (thrombokinase) acts upon the precursor prothrombin in the presence of calcium to form the enzyme thrombin which transforms fibrinogen into fibrin. Vitamin K was said to be necessary for the formation of prothrombin.

To-day the concept of blood coagulation is more complicated. It is still assumed that the process occurs in two stages: the formation of thrombin from its precursor prothombin and the transformation of fibrinogen into fibrin by means of thrombin. Thromboplastin is believed (OWREN and AAS, 1951) to act upon a "new" plasmatic factor "proconvertin" (KOLLER's factor VII) to form "convertin" which converts prothrombin into thrombin. This latter process is accelerated by means of "accelerin" formed from a plasmatic factor "pro-accelerin" by the action of thrombin.

Before the advent of proconvertin DAM and SØNDERGAARD (1948) showed that at least two plasmatic coagulation factors are lacking in vitamin K

deficiency. In the just mentioned terminology this will mean, for reasons to be described later, that vitamin K is necessary for the formation of prothrombin and proconvertin.

(*d*) *Where the action takes place*

That the action of vitamin K takes place in the liver has long been assumed, since liver damage or exclusion of the liver from the circulation causes a fall in prothrombin activity* which cannot be remedied with vitamin K.

Recently, LASCH and ROKA (1953) have reported that liver mitochondria form prothrombin, preferably at *p*H 7·9 and in an atmosphere of oxygen. They further report that the yield of prothrombin depends upon the amounts of proconvertin ("factor VII") and vitamin K available. The amount of proconvertin should decrease as prothrombin is formed. These reports still await confirmation. It seems somewhat surprising that the amount of prothrombin formed, but not the velocity of its formation, is said to increase with the amount of vitamin K present.

(*e*) *Antagonism between vitamin K and anticoagulants of the dicoumarol type*

Low prothrombin activity is produced by derivatives of 3-hydroxy-1,4-naphthoquinone, hydroxycoumarine and indanedione containing the configuration —OC.(R)C=C(OH)— depending upon the nature of the radical R. This characterization of the structure of anticoagulants of the dicoumarol type is due to MENTZER (1948) and SMITH (1950).

MANN, MANN, and BOLLMAN (1950) found that under given conditions, in rats, dicoumarol decreased proconvertin (their "co-thromboplastin") much more than prothrombin, while vitamin K deficiency produced a greater decrease in prothrombin than in proconvertin ("co-thromboplastin"). This is in agreement with earlier experiments of DAM and SØNDERGAARD (1948) in which it was found that mixtures of vitamin K deficient chicken plasma and plasma from dicoumarol poisoned chicks had a shorter prothrombin time than the two plasmas. Thus, it may be inferred that vitamin K deficient chicken plasma is predominantly lacking in prothrombin, whereas plasma from chicks poisoned with dicoumarol, under the conditions chosen, is predominantly lacking in proconvertin. By suitable adjustments of the experimental conditions, it is possible, however, to obtain dicoumarol chicken plasma which does not shorten the prothrombin time of the plasma from vitamin K deficient chicks (DAM and SØNDERGAARD, 1954, in press).

A predominant decrease of proconvertin in dicoumarol treatment has also been found by OWREN and AAS (1951) and by KOLLER *et al.* (factor VII) (1951).

It must be remembered that the current formulation of the coagulation process has no claim to be considered final, and it may very well be that the explanation of the just mentioned findings is more complicated. Experiments by SØRBYE, KRUSE, and DAM (1950) point in this direction.

* The term "prothrombin activity" means the combined activity of the various plasmatic factors which determine the prothrombin time.

It has been known for some time that vitamin K_1 and vitamin K_1 oxide given in sufficient doses may neutralize the effect of anticoagulants of the dicoumarol type, whereas the simpler vitamin K substitutes such as menadione, synkavite, etc., have little effect in this respect. Isler *et al.* (1953) have studied the efficiency against dicoumarol, etc., of vitamin K_1, vitamin K_2 and a series of their analogues with shorter or longer side chains. They found that the side chain in the 3 position must contain at least 8 carbon atoms. The methyl groups in the side chain as well as the double bond(s) in the same are favourable but not absolutely necessary.

The reason why the simple, otherwise highly active, vitamin K active compounds of the menadione type cannot neutralize the action of dicoumarol anticoagulants is not quite clear. It is likely that the phenomenon is related to the fact that these compounds act slower than vitamin K_1 in the vitamin K deficient chick.

The establishment of the fact that overdosage with the naturally occurring forms of vitamin K can overcome the action of dicoumarol, etc., has strengthened the conception that these anti-coagulants act by displacing vitamin K from an enzyme system involved in the formation of the prothrombin active plasma factors (prothrombin and proconvertin). Thereby some light has been thrown upon the mode of action of vitamin K itself. Quick and Collentine (1950, 1951) have discussed the displacement hypothesis further. They suggest that vitamin K_1 is more firmly associated with the apoenzyme than is menadione.

Studies in which dicoumarol labelled with C-14 in the methylene bridge was administered intravenously to mice and rabbits (Spinks and Jaques, 1950 Lee, Trevoy, Spinks, and Jaques, 1950) have shown that after 24 hr about 10 per cent had been fixed in the liver as structurally unchanged dicoumarol. The period during which dicoumarol remained in the liver was related to the effectiveness of this agent in interfering with the formation of the prothrombin active factors. Indirectly, this observation lends support to the concept that vitamin K exerts its action through the liver.

From the older literature (e.g. Dam, Glavind, Lewis, and Tage-Hansen, 1938) it is known that the liver does not store large amounts of vitamin K. When, however, a large dose of an aqueous colloidal solution of vitamin K_1 is injected intravenously (Dam, Prange, and Søndergaard, 1954, in press), it is easy to demonstrate a very considerable accumulation of vitamin K_1 in liver and spleen. The concentration of the injected vitamin in the blood declines rapidly, so that it cannot be determined by the Irreverre and Sullivan reaction after about 14 hr. The vitamin injected remains for a considerable time in the liver and spleen.

When at various time intervals after the injection of a large dose of vitamin K_1 a dose of a dicoumarol anticoagulant is given the anticoagulant effect is almost nil when the time interval is only one or a few days, but as the time interval becomes longer (several weeks) the effect of ingested anticoagulant increases gradually. After 5 weeks, when the animal still contains a considerable amount of vitamin

K_1, the prothrombin activity yields very markedly to ingestion of anticoagulant. It is therefore possible that the amount of vitamin K_1 present at this stage is deposited in such a way that it cannot easily be utilized. When large doses of vitamin K_1 are given orally or intramuscularly, even in aqueous colloidal solution, a similar deposition is not obtained.

C-14 labelled menadione given intravenously in large doses disappeared rapidly from the blood without being deposited, except for traces in liver and lung (Solvonuk *et al.*, 1952).

Martius (1953) and Martius and Nitz-Litzow (1953) have found that dicoumarol and related substances depress aerobic phosphorylation in rat liver mitochondria, and suggest that dicoumarol anticoagulants interfere with a complex with vitamin K operating between cytochromes b and c.

Since, however, vitamin K deficiency has hitherto only been shown to lead to deficiency in prothrombin-active plasma factors and not to a metabolic disturbance of a more general character reflected in slower growth, this theory needs further experimental proof. It seems almost certain that dicoumarol possesses toxic properties quite unrelated to its interference with the blood clotting mechanism. This applies, for instance, to the appearance of convulsions after large doses leading to death before the haemorrhagic tendency has become manifest. The same toxic effects have not been found for all anticoagulants of this type and have never been observed in alimentary vitamin K deficiency.

(*f*) *The route of absorption from the intestine*

Mann, Mann, Bollman, and Van Hook (1949, 1950) have shown that vitamin K deficiency (in rats) can be produced rapidly by external drainage of the lymph. This observation indicates that the natural fat-soluble K-vitamins are transported (largely) by the lymph from the intestines to the blood.

Millar, Leddy, and Fisher (1953) administered radioactive vitamin K_1 (2-methyl-C^{14}-3-phytyl-1,4-naphthoquinone) orally to rats with intestinal lymphatic fistula. They found that a greater proportion of the radioactivity appeared in the intestinal lymph, some appeared in the urine. Of radioactive menadione given orally to rats or dogs with intestinal lymph fistula only about 1 per cent appeared in the intestinal lymph, while 40 to 50 per cent of the ingested radioactivity was excreted in the urine and biliary fistula, large amounts of radioactivity from ingested radioactive menadione appeared in bile and urine very quickly. Exclusion of bile from the intestine did not alter the absorption pattern, but about 3 per cent of the administered activity appeared in the faeces of such animals. In experiments with cats it was shown that 74 per cent of an oral dose of menadione was absorbed into the portal system in 5 hr.

(*g*) *Vitamin K deficiency accelerated by sulphonamide derivatives and antibiotics*

Since intestinal bacteria form vitamin K (probably K_2) it is obvious that depression of the bacterial flora by sulphonamides or antibiotics may hasten

the development of the symptoms of vitamin K deficiency provided the diet does not furnish sufficient vitamin K.

Observation of this effect has been published by KORNBERG *et al.* (1944a,b) and by GRANADOS and DAM (1945) regarding sulphadiazine fed to rats. Unpublished experiments from the reviewer's laboratory have shown that in chicks raised on a vitamin K deficient diet the development of haemorrhages may be hastened somewhat by incorporating 10 mg aureomycin per 100 gm of the diet. Counts of bacteria in various parts of the intestine revealed great individual changes, but no significant decrease of the flora resulting from the addition of aureomycin, but the chicks grew faster with aureomycin. It cannot be excluded, therefore, that in this experiment the faster development of the symptoms was due to the more rapid growth.

(*h*) *The haemorrhagic condition in hypervitaminosis A*

The haemorrhagic condition, which is part of the hypervitaminosis A syndrome (RODAHL and MOORE, 1943; MOORE and WANG, 1945; RODAHL, 1949, 1950) has been found by LIGHT, ALSCHER, and FREY (1944) (in rats) to be due to low prothrombin activity, which could be raised by relatively small amounts of menadione. WALKER, EYLENBURG, and MOORE (1947) obtained the same result with Synkavit. The way in which vitamin A in large doses produces "hyperprothrombinemia" has not been completely elucidated. It seems, however, that the diets used were low in vitamin K, so that it is possible that vitamin K supply from the intestinal flora was impaired. RODAHL (1950) did not describe haemorrhage as part of the syndrome in chicks, and QUICK and STEFANINI (1948) could not produce prolonged prothrombin times in chicks by overdosing with vitamin A. This has been confirmed in the author's laboratory (unpublished).

(*i*) *Vitamin K deficiency symptoms in rats fed dihydroxystearic acid*

LOCKHART *et al.* (1942) found that the feeding of 25 per cent synthetic fat containing dihydroxystearic acid (1 mol of this acid + 2 mol of other fatty acids in the triglyceride) in vitamin K-deficient rat diets resulted in rapid development of vitamin K deficiency symptoms. Small amounts of menadione prevented the symptoms. This observation was further investigated by NIGHTINGALE *et al.* (1947). They found that dihydroxystearic acid blocks the synthesis of vitamin K by intestinal bacteria without killing the bacteria.

(*j*) *Requirement in chicks and dogs*

Older experiments from the author's laboratory (DAM and GLAVIND, 1938) indicate that 0·7 per cent of dried alfalfa in an otherwise vitamin K free diet for chicks will represent the approximate limit for maintaining normal prothrombin activity. This would correspond to about 16 to 20 μgm of vitamin K_1 per 100 gm diet.

ALMQUIST, MECCHI, and KLOSE (1938) found that an amount of alfalfa extract corresponding to 1 per cent dried alfalfa in the diet would ensure normal

clotting time in 3-week-old chicks, whereas an amount corresponding to 0·5 per cent gave somewhat higher clotting times. CRAVENS, RANDLE, ELVEHJEM, and HALPIN (1941) indicate that 2 per cent of dried alfalfa will maintain normal clotting time in small chicks. This, however, is above the limit. QUICK and STEFANINI (1948) found that 1·5 μgm of menadione or little more than 2·5 μgm of vitamin K_1 per day are required to maintain the prothrombin level of a chick during the first 10 days of life. In the curative assay (single dose, 20 hr test) by DAM, KRUSE, and SØNDERGAARD (1951) about 0·03 mg menadione per kgm body weight given orally to chicks will lower the prothrombin time to normal value. QUICK, HUSSEY, and COLLENTINE (1954) found for *dogs* with a cholecystonephrostomy that approximately 0·5 μgm of K_1 per kgm of body weight per day, given intravenously, would maintain normal prothrombin level.

(*k*) *Vitamin K in the human*

The *requirement* for vitamin K of the human adult is not exactly known. In newborn it has been shown (SELLS, WALKER, and OWEN, 1941; HARDWICKE, 1944; LARSEN, 1952) that an amount of 1 to 10 μgm of one of the artificial vitamin K substitutes ("K_S" or Synkavit) per day will suffice to maintain normal prothrombin activity. It must be remembered that part of the supply of vitamin K is believed to originate from the intestinal flora. In disorders in adults, associated with low prothrombin activity amendable by ingestion of vitamin K, a daily supply of a few milligrams of Synkavit, etc., will suffice, and, probably, is considerably above the necessary limit.

FANTL, NELSON, and LINCOLN (1951) have proposed a saturation test for vitamin K deficiency depending on the oral administration of the diacetate of 2-methyl-1,4-naphthoquinone (acetomenaphthone) and estimation of the urinary excretion.

(*l*) *The bleeding tendency associated with obstructive jaundice*

This earliest known form of vitamin K deficiency in man has been suspected of being complicated by the presence of increased antithrombin over and above lowered prothrombin activity (MARX and DYCKERHOFF, 1943).

OWREN (1949) found some cases of obstructive jaundice without liver damage to have an increased amount of proaccelerin (factor 5), others had normal amounts. Proaccelerin was not dependent upon the supply of vitamin K. Parenteral administration of 50 to 100 mg of menadione produced a rapid increase of the "prothrombin" concentration to normal or supernormal levels. (This was before OWREN and BJERKELUND (1949) and OWREN (1951) distinguished between prothrombin and proconvertin.)

KOLLER, LOELIGER, DUCKERT, and HU-WANG (1952) find both factor 7 (proconvertin) and prothrombin low in obstructive jaundice. Vitamin K increased factor 7 particularly.

It has been shown that in addition to the common water-soluble menadione derivatives and menadione itself also acetomenaphthone (FANTL, NELSON, and LINCOLN, 1951) and large doses of vitamin K_1 (QUICK, HUSSEY, and

COLLENTINE, 1954) (with dogs) given as aqueous emulsion may be absorbed from the intestine in the absence of bile.

(*m*) *The diagnostic application of vitamin K*

Viz. for distinguishing between obstructive jaundice and hepatitis was discussed in the earlier review (DAM, 1948). Recent contributions are, for instance, the above-mentioned publication by OWREN (1949) taking also proaccelerin (factor 5) into consideration. FORELL and KOLLER (1953) determined prothrombin, proconvertin (factor 7) and proaccelerin. In accord with the findings of OWREN, proaccelerin was found uninfluenced by vitamin K. DE NICOLA (1953) found Ac-globulin (proaccelerin) diminished besides proconvertin and prothrombin in mild hepatic disorders and in some cases of obstructive jaundice complicated by liver damage. Vitamin K_1 or vitamin K_1 oxide were reported also to increase proaccelerin.

(*n*) *The haemorrhagic disease of the newborn*

At the university hospital in Copenhagen, LARSEN (1952) and DYGGVE (1952) have carried out extensive studies on this subject. LARSEN studied the alterations in prothrombin activity of the newborn, DYGGVE the value of vitamin K treatment.

LARSEN has shown that the lowered prothrombin activity normally present immediately at birth is not influenced by vitamin K treatment of the mother *ante partum*. Therefore, the relativity low prothrombin activity at birth cannot be due to vitamin K deficiency; other as yet unknown factors of a physiological or a chemical nature must account for the limited production of "prothrombin" in uterine life. Extremely low prothrombin activity has been found in asphyctic newborn.

At birth the child has no stores of vitamin K to prevent the fall of prothrombin activity in the first days of the first week. It is this decline in prothrombin activity after birth that is prevented by treatment with vitamin K either of the mother *ante partum* or of the child immediately after birth. The decline after birth can also be prevented by giving the child cow's milk instead of breast milk in the first week. It is held that it is the small amount of vitamin K in the milk that raises the prothrombin activity rather than vitamin K formed by the intestinal flora.

It is a somewhat puzzling fact that during foetal life there is just enough vitamin K to allow the production of the amount of "prothrombin" possible in the physiological circumstances, but unless the mother has been supplied with a large excess, not enough to furnish the child with the small store of vitamin K that could prevent the fall in the first week after delivery. LARSEN (1952) has tentatively suggested that in foetal life "prothrombin" might be formed in the placenta and not in the liver. This hypothesis would explain the complicated phenomenon, but there is no direct evidence in support of it.

The fact that the prothrombin activity of the infant does not reach the value for normal adults until about 6 months of age, even with sufficient vitamin K

supply, must mean that the production of prothrombin or of some of the auxiliary factors is fixed at a lower limit than in later stages of life. The reason for this is as yet unexplained. However, since there is a limit to the amount of prothrombin and auxiliary factors in adult life also, it is not particularly surprising that the limit is not the same at all stages of life. In the later stages of pregnancy, for instance, the limit is higher than at other times.

KOLLER and HELD (1952) have made separate determinations of prothrombin and their factor 7 in the newborn and in mothers during the later part of pregnancy. They found that in the newborn both are about equally depressed. In the later part of pregnancy the amount of prothrombin of the mothers is almost unchanged, while that of proconvertin is increased. This may account for the apparent hyperprothrombinemia found by methods that do not distinguish between prothrombin and proconvertin.

Attempts to distinguish between the various coagulation factors concerned with the low prothrombin activity of the newborn have also been made by RANDALL and RANDALL (1949) and OWEN and HURN (1953).

Some authors have reported a seasonal variation in the prothrombin activity of the newborn (WADDELL and LAWSON, 1940; SNELLING and NELSON, 1943, and others). PLUM (1949) has examined a large number of cases in Copenhagen throughout the year without being able to find any variation significantly related to season. In this kind of investigation it is necessary always to compare prothrombin activities at the same time after birth, preferably the third or fourth day, when the minimum occurs.

There is, however, a seasonal variation in the frequency of haemorrhages (especially melaena) in the newborn of untreated mothers. This has been found by several investigators in various parts of the world. The incidence is minimal in late summer and early spring. Taken together with the apparent absence in seasonal variation in prothrombin times, this must mean that other factors besides prothrombin activity decide whether haemorrhages will occur or not.

(o) *Value of vitamin K treatment of the mothers*

DYGGVE (1952) studied the occurrence of various forms of haemorrhages in 22,371 newborn from untreated mothers and 10,879 newborn from mothers treated with Synkavit or a few other artificial vitamin K substitutes 24 to 1 hr before delivery. There were 371 cases of various haemorrhages in 10,000 newborn in the untreated group and 276 in 10,000 in the group from treated mothers. Kephal haematomas (111 in 10,000 in the untreated group and 105 in 10,000 in the treated group) did not respond significantly to vitamin K treatment. The best results were obtained against umbilical haemorrhages, haemoperitoneum and melaena.

DYGGVE estimates that possibly 1 out of 1000 newborn dies from haemorrhage that can be prevented by vitamin K treatment of the mothers. In Denmark, with about 80,000 births per year, this means a total number of 80. The number of non-lethal haemorrhages preventable by the treatment is much greater.

It is interesting to compare DYGGVE's figures with the more optimistic estimates in the early era of vitamin K; when it was expected that vitamin K treatment would reduce the death rate of newborn infants much more (HELLMAN and SHETTLES, 1942, and others) and the entirely pessimistic, but insufficiently founded statement of SANFORD, KOSTALIK, and BLACKMORE (1949) who claimed that vitamin K treatment of the newborn had no value at all (cf. DAM, 1951).

Another discussion on the advisability of maternal vitamin K prophylaxis against haemorrhagic disease of the newborn is found in *Lancet* (1951, HAY, HUDSON, and RODGERS, 1951, DYGGVE and PLUM, 1951).

(*p*) *Use in anticoagulant therapy*

If, in anticoagulant therapy with dicoumarol and its analogues the prothrombin activity decreases so much that haemorrhages may occur (e.g. below 10 per cent of OWREN's "P-P-value") (OWREN and AAS, 1951), vitamin K must be given in a form which will counteract the anticoagulant rapidly. For this purpose vitamin K_1 or its oxide are the substances to use, whereas menadione, Synkayvite and similar simpler vitamin K-active substances are insufficient.

In order to obtain a rapid action it is necessary to give, for instance, vitamin K_1 intravenously in the form of a sterile colloidal solution in water. The amount to inject should not be too great if it is not intended to raise the prothrombin activity to the normal value. Ten to 20 mg or less may be an appropriate dose with which values within the therapeutic interval (10 to 30 per cent of OWREN's P-P-value) may be reached in 1 or 2 hr. A comprehensive literature on this aspect of vitamin K treatment exists, cf. for instance, DAM, GEILL, LUND, and SØNDERGAARD (1954).

(*q*) *Vitamin K in therapy with antibiotics*

Treatment with antibiotics or with sulphonamides aiming at the depression of the bacterial flora in the intestine ought to be accompanied by vitamin K treatment.

(*r*) *Effects of naphthoquinones unrelated to vitamin K activity*

Of such effects shall be mentioned a general bacteriostasis (ARMSTRONG, SPINK, and KAHNKE, 1943; FOX, 1953), inhibition of alcoholic fermentation especially by "K_5" (PRATT, SAH, DUFRENOY, and PICKERING, 1948), and interference with mitosis (LEHMANN, LÜSCHER, and HUBER, 1945; NEUKOMM, GUIRON, LERCH, and RICHARD, 1953).

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V. GUINEA PIG ANTISTIFFNESS FACTOR

Stiffness of joints, e.g. wrist stiffness in guinea pigs, was found by WULZEN and BAHRS (1941) using diets of skim milk fortified with skim milk powder. Later, the disease was reported in guinea pigs fed other diets (e.g. OLESON, VAN DONK, BERNSTEIN, DORFMAN, and SUBBAROW, 1947).

The disease produced with the skim milk diet was associated with abnormal calcification, e.g. calcification of skeletal muscles and several other tissues (HARRIS and WULZEN, 1950). Calcification of the auditory complex may lead to deafness (WULZEN and PLYMPTON, 1949a,b; KRUEGER, WULZEN, and LEVEQUE, 1950). Changes occurred in the distribution of acid-soluble phosphorus in liver and kidneys (VAN WAGTENDONK, 1944). Alkaline serum phosphatase was lowered (VAN WAGTENDONK, RATHKEY, BALLOU, and WULZEN, 1944). Inorganic phosphate and total calcium in blood were elevated (VAN WAGTENDONK, FREED, and BALLOU, 1944). A factor which cures an induced stiffness was reported to be present in raw cream (VAN WAGTENDONK and WULZEN, 1943). Later interest was focused on a factor in raw sugar cane juice which was isolated in crystalline form (VAN WAGTENDONK and WULZEN, 1946) and described as a steroid compound (ROSS, VAN WAGTENDONK, and WULZEN, 1949). This compound was identified as stigmasterol (KAISER and WULZEN, 1951; ROSENKRANTS, MILHORAT, FARBER, and MILMAN, 1951).

OLESON *et al.* (1947) found ergostanyl acetate to be active. This was confirmed by PETERING, STUBBERFIELD, and DELOR (1948) although much higher doses had to be used in their experiments. DASLER and BAUER (1949) could not confirm the antistiffness activity of ergostanyl acetate, and DASLER (1950) criticized the assay method.

The possible relation to collagen diseases in man has been mentioned by LANSBURY, SMITH, WULZEN, and VAN WAGTENDONK (1950).

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VI. THE ANTIGIZZARD ULCER FACTOR

The factor which prevents gizzard ulcers in chicks is not a single substance. At least one fat soluble and one water soluble substance are required to prevent this symptom. Highly unsaturated fatty acids from hog liver, or an ether extract from calves brain, may act as the fat soluble, whereas vitamin B_{12} may function as the water soluble factor. Since no great development seems to have been published in this field since the author reviewed the subject in *Annual Review of Biochemistry* (1951) the reader is referred to this article.

REFERENCE

DAM, H. (1951) *Annual Review of Biochemistry*, **20**, 265.

6

OXYGENATED FATTY ACIDS

Daniel Swern

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6

OXYGENATED FATTY ACIDS

Daniel Swern

I. Introduction

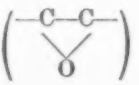
Although non-oxygenated fatty acids have been known and characterized since the early part of the nineteenth century, oxygenated fatty acids, with perhaps one notable exception (ricinoleic acid), were first characterized at the end of the nineteenth century, and they have been studied extensively and systematically only during the past twenty-five years. Oxygenated fatty acids have not yet achieved the industrial importance of non-oxygenated fatty acids, mainly because of their relative unavailability either from natural sources or synthetically. But interest in oxygenated fatty acids and applications for them continue to increase rapidly as more of their useful properties become better known and more efficient and inexpensive synthetic methods are developed for their preparation.

Oxygenated fatty acids are essential intermediates in a variety of basic studies in fatty acid chemistry, notably autoxidation, chemical oxidation, and reaction mechanism and metabolism investigations, and they are important in industrial processes concerned with the oxidation and drying of oils. Oxygenated fatty acids are also attractive substances for studies of position, geometrical and optical isomerism, and for studies of the effect of position, type and number of functional groups on physical and chemical properties. Most oxygenated fatty acids are higher melting and easier to crystallize than their corresponding non-oxygenated or unsaturated analogues, thus rendering them relatively easy to isolate and study. The opportunities which exist for isomerism, however, require that extreme care be taken in their preparation and isolation and in selecting criteria of purity.

In this chapter, both naturally occurring and synthetically prepared oxygenated fatty acids will be discussed. The emphasis, naturally, will be on those compounds containing eighteen carbon atoms since these exceed in importance by far all other oxygenated fatty acids combined. The classes of oxygenated fatty acids to be considered are epoxy acids, hydroxy acids and keto acids. The discussion will include their occurrence and isolation from natural sources or methods of preparation, physical and chemical characteristics, commercial utility, if any, and application to isomerism and structure studies.

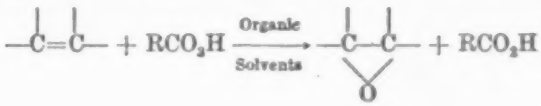
II. Epoxy Acids

(*a*) *Preparation*

Epoxy fatty acids are straight-chain aliphatic monocarboxylic acids containing one or more oxirane groups 

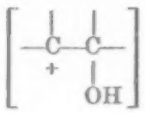
in the fatty acid chain. This class of compounds does not occur in nature. Occasionally the isolation of epoxy acids from fats is reported, but in all probability the fat was not fresh and had undergone autoxidative changes.

The most important and most widely used method for the preparation of epoxy fatty acids is epoxidation of the corresponding olefinic acid with organic peracids, notably perbenzoic, peracetic and monoperphthalic acids:[1], [2]

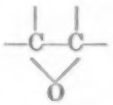


In some cases performic acid can be used. The reaction is rapid at or below room temperature, it proceeds in a homogeneous system, the product is easy to isolate and, in general, yields are good, frequently exceeding 80 per cent.

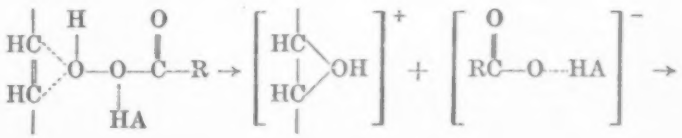
The epoxidation reaction is strikingly stereospecific, that is, the geometrical configuration of the resulting epoxy acid is identical with that of the olefinic acid from which it is derived. This high degree of stereospecificity precludes a free carbonium ion intermediate involving attack of the double bond by the simple electropositively polarized (electrophilic) hydroxyl group[3] $[\ddot{\underset{\cdot\cdot}{O}}:H]_+$ to yield



which on loss of a proton and ring closure yields

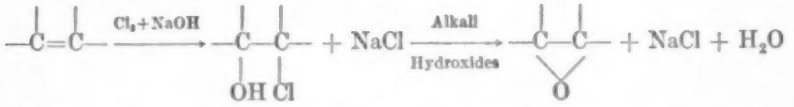


Since epoxidation is subject to general acid (HA) catalysis,[4], [5] a more reasonable mechanism for the reaction would involve essentially direct formation of the conjugate acid of the oxirane by donation of $[\ddot{\underset{\cdot\cdot}{O}}:H]^+$ to the double bond by a peracid-general acid complex:[1]



The olefin $-\left[\ddot{\underset{\cdot\cdot}{O}} : H\right]^+$ part of the transition state in such a process is similar to the so-called π-complexes.[6] An attractive feature of this mechanism over that involving free $\left[\ddot{\underset{\cdot\cdot}{O}} : H\right]^+$ is that it obviates any necessity for postulation of rapid and reversible $\left[\ddot{\underset{\cdot\cdot}{O}} : H\right]^+$ formation from peracid and general acid (HA) followed by slow attack of $\left[\ddot{\underset{\cdot\cdot}{O}} : H\right]^+$ on the double bond. It is also a more reasonable path in the non-polar solvents often used as epoxidation reaction media.

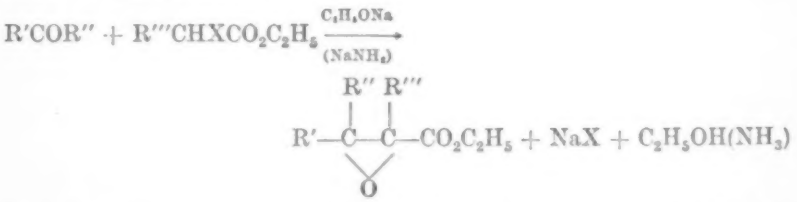
The earliest method for the preparation of epoxy acids, and one which is still used to some extent, is hypochlorination of the olefinic acid followed by dehydrohalogenation:[7], [8], [9]



$$-C=C- \xrightarrow{Cl_2+NaOH} -C(OH)-C(Cl)- + NaCl \xrightarrow[Hydroxides]{Alkali} -C(O)-C- + NaCl + H_2O$$

The chlorohydrins can be prepared in substantially quantitative yield,[10] but in large-scale experiments the dehydrohalogenation to the oxirane usually proceeds with difficulty and side reactions occur so that the overall yield of epoxy acids rarely exceeds 50 per cent. In small-scale experiments, however, excellent yields of epoxide can be obtained.[11]

α,β-Epoxy esters can be prepared in fair to good yields by the Darzens condensation,[12] which involves the reaction of an aldehyde or ketone with an α-halo ester in the presence of alkaline condensing agents, particularly sodium ethoxide or sodium amide:



This reaction has been explored to only a limited extent with aliphatic aldehydes and ketones containing more than five carbon atoms or with α-halo esters of high molecular weight acids.

Autoxidation of olefinic acids gives small yields of epoxy acids generally not exceeding 15 per cent. The difficulty in isolating the products, however, renders this method of little value.

Although many epoxy acids and esters have been prepared, the two most important members of this class and those which have been characterized the best are *cis*- and *trans*-9,10-epoxystearic acids, m.p. 59·5° and 55·5°, respectively, prepared from oleic or elaidic acid by epoxidation.[1], [2]

(*b*) *Properties and reactions*

Epoxy acids are white crystalline solids which have melting points appreciably higher than those of the parent unsaturated acids. They are generally quite

soluble in the common organic solvents at or above room temperature, and are conveniently recrystallized from acetone, methanol or hexane. In general, epoxy acids show the typical reactions of ethylene oxide, that is, they react with many classes of compounds containing active hydrogen atoms, such as alcohols, amines, and acids. In the reaction of epoxy acids with alcohols in the presence of acid catalysts, the carboxyl group is esterified simultaneously with the ring-opening reaction to yield a hydroxy-ether-ester.[13] Also, since monoepoxy acids are bifunctional, those with the oxirane group in suitable positions can be readily converted to high molecular weight polyesters on heating.[14] Hydrogenation, either catalytically[15], [16] or with lithium aluminium hydride,[17] converts the oxirane to the hydroxyl group. In the former reaction with 9,10-epoxystearic acid, it is believed that the resulting mixture of monohydroxy acids contains the 10-isomer to the extent of at least 85 per cent.[15] Alkaline or acidic hydrolysis of epoxy acids converts the oxirane group to the α-glycol group with inversion.[18], [19] Reaction of epoxy acids with carboxylic acids opens the oxirane ring and yields hydroxy-esters or diesters which can be thermally dehydrated and deacylated to conjugated dienes.[20] Reaction of epoxy acids with ammonia or amines yields long-chain amino acids also containing a hydroxyl group.[21]

Epoxy acids exhibit an interesting type of mixed stereoisomerism, existing both as optical as well as geometrical isomers. Thus, 9,10-epoxystearic acid contains two asymmetric carbon atoms, thereby furnishing two racemic mixtures, the high-melting (59·5°) and low-melting (55·5°) forms. In addition, because of the inability to undergo free rotation as a result of the oxirane group, these racemic mixtures are also geometrical (*cis-* and *trans-*) isomers. Recent x-ray diffraction studies have shown that the high-melting racemic mixture is *cis-* and the low-melting is *trans-*.[22]

The infrared spectra of *cis* and *trans* epoxy fatty acids are substantially the same except that the *cis* isomer shows a characteristic absorption near 12 microns caused by the oxirane ring, whereas the *trans* isomer shows the characteristic absorption near 11·2 microns. Determination of infrared spectra and x-ray diffraction patterns, therefore, permit unequivocal identification of the *cis-* and *trans*-9,10-epoxystearic acids.[23] Furthermore, these physical measurements require only small samples and little time.

Industrial uses for the epoxy acids are limited. It has been shown recently that esters of epoxy acids are useful plasticizers and hydrogen chloride acceptors for use in polyvinyl chloride.[24] The neutralization of hydrochloric acid evolved when polyvinyl chloride is exposed to heat and/or light prevents the development of undesirable colour and rapid deterioration of the polymer. Epoxy esters are frequently referred to as stabilizing plasticizers for chlorine-containing polymers and they are used on a large industrial scale. The avidity with which epoxy fatty acids and their derivatives react with hydrogen chloride is the basis of an analytical method for their quantitative determination.[25] Epoxy acids are also cleaved by periodic acid, presumably because the oxirane is converted to the α-glycol by hydrolysis.

III. Monohydroxy Acids

The monohydroxy fatty acids are straight-chain aliphatic monocarboxylic acids containing one hydroxyl group in the aliphatic chain. They may be either saturated or unsaturated. The naturally occurring hydroxy acids are optically active whereas the synthetic ones are inactive and are capable of being separated into optical isomers. The unsaturated hydroxy acids exhibit both optical and geometrical isomerism.

Generally speaking, saturated monohydroxy fatty acids do not occur to any significant extent as component acids of fats, waxes, and related materials. The waxy substances from leaves of certain coniferous plants, however, contain significant amounts of 12-hydroxydodecanoic (sabinic) acid and 16-hydroxyhexadecanoic (juniperic) acid.[26], [27] Hydroxy fatty acids, such as 2-hydroxytetradecanoic and 2-hydroxyhexadecanoic acid, are present as components of wool wax.[28] Higher homologues are also assumed to be present. Brain lipids, notably the cerebrosides, contain 2-hydroxy acids of the C_{22}, C_{24} and C_{26} straight-chain series, with the C_{24} acid predominating.[29], [30], [31] The presence of relatively large amounts of hydroxy fatty acids in brain lipids is a characteristic difference between the component fatty acids of brain lipids and the fatty acids present in animal fats. The biological function of the hydroxy acids in brain lipids is unknown and is of considerable fundamental and biochemical interest.

A monohydroxytetradecanoic acid has been isolated from the oil of *Angelica archangelica*.[32] Convolvulinolic acid, 11-hydroxypentadecanoic acid, has been isolated from the roots of plants of the *Convolvulaceae* family.[33] This acid is unusual since it contains an odd number of carbon atoms. An optically active monohydroxyhexadecanoic acid has been isolated from butter fat, but its structure has not been established.[34] Hydroxy fatty acids have also been isolated from the waxes of human and bovine tubercle baccilli,[35], [36], [37] and leprosy bacilli.[38] High molecular weight fatty acids are reported to be present in the root fat of *Swertia japonica*,[39] in the hydrolysis products of cork[40], [41] and in kidney lipids.[42]

2-Hydroxy acids are important intermediates in biological oxidation of fatty acids and are also synthesized during insect and plant metabolism. Since the hydroxy acids are intermediates in many metabolic processes, they would not be expected to be present in large amounts.

(*a*) *Preparation of saturated monohydroxy acids*

The classical method for preparing monohydroxy fatty acids is hydrolysis of monohalogenated fatty acids with aqueous alkaline solutions.[31] Halogenated fatty acids with the halogen originally in the 4- or 5- position readily form lactones on acidification of the soap solutions of the resulting hydroxy acids, especially if heated.

A convenient procedure for preparing monohydroxy compounds of known structure is hydrogenation or chemical reduction (with lithium aluminium hydride, for example) of the corresponding oxirane[15], [16], [17] or keto compound.[43] No more than two isomers are apparently obtained from the oxirane,

and recent evidence indicates that the isomer which predominates is the one in which the hydroxyl group is farther from the carboxyl group.[15] As already discussed, the oxirane compounds can be conveniently prepared by epoxidation of olefinic acids with organic peracids. Keto acids of known structure can be prepared synthetically from shorter-chain intermediates by a variety of condensation reactions,[43] as will be discussed in a later section. By high pressure hydrogenation of the methyl esters of the corresponding keto acids, 3- through 17-monohydroxyoctadecanoic acids have recently been prepared.[43] The 2-isomer was prepared by alkaline hydrolysis of 2-bromooctadecanoic acid and the 18-isomer was prepared by hydrogenolyses of the monothiol ester of octadecane-1,18-dioic acid. The polymorphism of these, as well as the corresponding keto compounds, has been studied by thermal and x-ray methods.[43] It is now possible to identify unequivocally any isomeric monohydroxy or monoketo-octadecanoic acid or their methyl esters. The hydroxy compounds are more complicated with regard to polymorphic behaviour than the keto compounds.

Reaction of olefinic acids with concentrated sulphuric acid at low temperatures followed by hydrolysis of the intermediate sulphate esters also yields hydroxy acids, but complicated mixtures of isomers are obtained.[44], [45]

Autoxidation of olefinic acids to α-hydroperoxides followed by complete hydrogenation has been reported for the preparation of saturated hydroxy acids.[46] Until good yields of specific hydroperoxides can be obtained by autoxidation, this procedure will be of only limited interest. Further, this procedure yields a mixture of isomeric hydroxy acids.[46] Partial reduction of α-hydroperoxido acids yields hydroxy unsaturated acids.[46]

A newly discovered general reaction for the synthesis of monohydroxy acids, and one which appears to have wide applicability both on a laboratory and commercial scale, is the addition of 90 to 100 per cent formic acid at its boiling point to olefinic acids, followed by hydrolysis of the intermediate formate ester.[47] The reaction is markedly accelerated by strongly acidic catalysts, notably perchloric and sulphuric acids and boron fluoride. Yields of monohydroxystearic acids of over 70 per cent can be obtained. Two isomeric hydroxy acids predominate since, in the absence of directing influences, formic acid would be expected to add equally on each side of the double bond. Since the reaction involves carbonium ion intermediates, other isomeric hydroxy acids are also formed, but in small amounts. In some instances, the isomeric monohydroxy acids can be readily separated.[47] Acetic acid in the presence of acid catalysts also adds to the double bond but yields of monohydroxy acids are only about 40 per cent.[47] When a hydroxyl group is in close proximity to the double bond, as in the case of methyl ricinoleate and crotyl alcohol, formic acid addition does not take place. The failure to react in these cases may be explained on the assumption that the electron-withdrawing influence of the hydroxyl group lowers the electron density of the double bond below the point where attack by certain electrophilic (carbonium) ions is possible.

9-, 10-, 11-, 12- and 13-hydroxyoctadecanoic acids have been synthesized by the Grignard reaction from ω-aldehydo aliphatic acids and alkyl magnesium

Table 1. Saturated monohydroxy fatty acids[a]

Systematic name	*M.P., °C*	*Methyl ester*	
		M.P.	*B.P., °C/mm.*
2-Hydroxydodecanoic	73–4°	43°[b]	
3-Hydroxydodecanoic	70–70·5°		
4-Hydroxydodecanoic	62·5–63·5°		
9-Hydroxydodecanoic	B.P. 156°/1		
12-Hydroxydodecanoic	84–85°	34–35°	164–166°/3
2-Methyl-2-hydroxyhendecanoic	46°		
2-Hydroxytridecanoic	78°		
4-Hydroxytridecanoic	66–66·5°		
9-Hydroxytridecanoic	49–51°		154–155°/3
13-Hydroxytridecanoic	79–79·5°	44°	
2-Hydroxytetradecanoic	81·5–82°		
3-Hydroxytetradecanoic	72–73°		
11-Hydroxytetradecanoic	51°		
14-Hydroxytetradecanoic	91–91·5°	47°	196–198°/10
2-Hydroxypentadecanoic	84·5°	48°[b]	
11-Hydroxypentadecanoic	63·5–64°	29–32°	166°/2
15-Hydroxypentadecanoic	84·5–85·2°	52–52·5°	180–182°/2
2-Hydroxyhexadecanoic	86–87°	59–60°	230–231°/5
3-Hydroxyhexadecanoic	83–83·5°		
11-Hydroxyhexadecanoic	68–69°	40·5–41·5°	183–186°/3
16-Hydroxyhexadecanoic	95°	55–55·5°	194–196°/2
2-Hydroxyheptadecanoic	89°		
17-Hydroxyheptadecanoic	87·5–88°	58·6–59°	210°/3
2-Methyl-16-hydroxyhexadecanoic	62–63°		
2-Hydroxyoctadecanoic	91°	66°; 62–63°[b]	
3-Hydroxyoctadecanoic	89°	51°	
4-Hydroxyoctadecanoic	87°	56–59°	
5-Hydroxyoctadecanoic	82°	52°	
6-Hydroxyoctadecanoic	83°	58°	
7-Hydroxyoctadecanoic	78°	51°; 37·5°[b]	
8-Hydroxyoctadecanoic	82°	55·5°	
9-Hydroxyoctadecanoic	75°	50·5°; 45–46°	212–216°/4
10-Hydroxyoctadecanoic	82°	54°	213–217°/4
11-Hydroxyoctadecanoic	77°	50°	204–206°/4
12-Hydroxyoctadecanoic (optically active)	82°	58–59°	202–204°/4
12-Hydroxyoctadecanoic (dl)	79°	53°	185–189°/2
13-Hydroxyoctadecanoic	77°	53°	
14-Hydroxyoctadecanoic	77°(γ); 80°(ε)	54·5°	
15-Hydroxyoctadecanoic	75·5°	59°	
16-Hydroxyoctadecanoic	78·5°	61°	
17-Hydroxyoctadecanoic	76·5°; 81°	64°	
18-Hydroxyoctadecanoic	99°	62·5°	

Table 1—continued

Systematic name	*M.P., °C*	*Methyl ester*	
		M.P.	*B.P., °C/mm*
2-Hydroxynonadecanoic	83–84°		
19-Hydroxynonadecanoic	91–91·5°	65·5–66°	
2-Hydroxyeicosanoic	91–92°	62–64°	
20-Hydroxyeicosanoic	97·4–97·8°	68–68·5°	
21-Hydroxyhenicosanoic	92·5–93°	70–71°	
2-Hydroxydocosanoic	98°	70–71°[b]	
13(14)-Hydroxydocosanoic	89–90°	53°	
2-Hydroxytetracosanoic	100°		
22-Hydroxytetracosanoic	95–96°		
2-Hydroxyhexacosanoic	86·5°		
2-Hydroxytriacontanoic	97·5°		
2-Hydroxytetratriacontanoic	109[c]		

[a] The data in this table have been compiled mainly from BEILSTEIN'S *Handbuch der Organischen Chemie*, Vol. III; DECEL, H. J.: *Lipids*, Vol. I, Interscience Publishers, New York, 1951; DOSS, M. P.: *Properties of the Principal Fats, Fatty Oils, Waxes, Fatty Acids and Their Salts*, The Texas Co., New York, 1952; RALSTON, A. W.: *Fatty Acids and Their Derivatives*, John Wiley & Sons, New York, 1948; MARKLEY, K. S.: *Fatty Acids*, Interscience Publishers, New York, 1947; the literature references given in the section of this chapter entitled "Monohydroxy Fatty Acids," and unpublished research carried out in the author's laboratory.
[b] Ethyl ester.

bromides. Thus, 9-hydroxystearic acid, m.p. 75°, can be prepared by condensing nonyl magnesium bromide with 9-aldehydononanoic acid followed by hydrolysis.[48]

α-Hydroxy acids can be prepared by way of the intermediate cyanhydrins by reaction of aldehydes with hydrogen cyanide and a basic catalyst, followed by hydrolysis. The unavailability of long-chain aliphatic aldehydes severely limits the utility of this reaction in the fatty acid field. α-Hydroxy acids can also be prepared from aliphatic acids by reaction with bromine and red phosphorus, followed by hydrolysis.[49]

β-Hydroxy acids can be readily prepared by the Reformatsky reaction.[50] This reaction involves the condensation of an aldehyde or ketone with an α-haloester in the presence of zinc. β-Hydroxyvaleric to β-hydroxystearic acid have been prepared in this way.[51], [52], [53], [54]

ω-Hydroxy acids of the formula $HO(CH_2)nCO_2H$ (where n is 7–20) have been prepared by CHUIT and co-workers[55], [56] by a variety of methods, most important of which are the reduction of monoesters of dibasic acids with sodium and alcohol, and the reaction of potassium cyanide or sodium malonic ester with the monobromohydrins of α,ω-glycols and subsequent hydrolysis to the hydroxy acids.

Other reactions have been reported for the preparation of specific monohydroxy fatty acids, but the ones described are the most important by far.

Table 1 lists the better known saturated hydroxy fatty acids containing twelve or more carbon atoms.

(*b*) *Reactions and properties of saturated monohydroxy acids*

The presence of the hydroxyl group enhances the water and alcohol solubility of hydroxy acids and greatly reduces solubility in hydrocarbons. Hydroxy acids can usually be cleanly separated from unsaturated and saturated acids by a single crystallization from hexane. Final purification is readily effected by recrystallization from alcohols, acetone or other polar solvents. Mixtures of monohydroxy acids are usually difficult to separate into pure components because of similarity in properties.

Hydroxy fatty acids show typical alcoholic and carboxylic reactivity. The bifunctional character of the molecule makes some of them interesting in thermal polycondensation reactions. 2-Hydroxy acids, however, yield aldehydes, water, carbon monoxide and/or lactides when heated. 3-Hydroxy acids dehydrate readily to mixtures of unsaturated acids, and 4- and 5-hydroxy acids readily form lactones. Hydroxy acids in which the hydroxyl is farther from the carboxyl group polymerize readily on heating to yield polymeric esters, or dehydrate to unsaturated acids when heated to higher temperatures. Intermolecular esters of hydroxy acids are commonly referred to as estolides.

Numerous esters of monohydroxy fatty acids have been prepared. These have been suggested for use as high-melting waxes and release agents for plastics, and as intermediates in the preparation of plasticizers and surface-active agents.[57], [58], [59], [60], [61] It has recently been reported that saturated monohydroxy acids are converted in fair yield to dicarboxylic acids on fusion with strong alkalis at 280 to 320°.[61] The lithium salt of 12-hydroxystearic acid is an important ingredient in the preparation of high-quality, all-purpose, lubricating greases.

(*c*) *Unsaturated monohydroxy acids*

Ricinoleic acid, 12-hydroxy-*cis*-9-octadecenoic acid, apparently isolated for the first time by SAALMÜLLER in 1848,[62] is the most important unsaturated hydroxy acid. It occurs as a glycerol ester in oils of the species *Ricinus*, and in castor oil (oil from the seed of *Ricinus communis*) it forms over 85 per cent of the component glycerides.[63] It has also been reported in ivory wood oil (47 per cent), oil of ergot, argemone oil, and others. Other hydroxy unsaturated acids, some isomeric with ricinoleic acid, have also been reported in grapeseed oil, quinceseed oil, the oil from *Strophanthus sarmentosus*[64] and brain lipids. The unsaturated hydroxy acid in brain lipids is reported to be 2-hydroxy-9-tetracosenoic acid.[65] A highly unsaturated monohydroxy fatty acid ($C_{18}H_{26}O_3$) has recently been isolated from boleko (isano) oil.[66] Its structure has not been elucidated but the hydroxyl group is reported to be in the 8-position.

Pure ricinoleic acid has the following physical characteristics:[67] m.p. 5·0°

(γ), 7·7° (α), and 16·0° (β); n_D^{25} 1·4703; d_4^{25} 0·9417; $[\alpha]_D^{25°}$ + 7·79°. Ricinoleic acid cannot be distilled satisfactorily because it readily undergoes dehydration and interesterification. The isolation of pure ricinoleic acid is extremely difficult and was achieved only recently by HAWKE,[68] who employed a modification of a published[69] low temperature solvent crystallization procedure used earlier in obtaining a purity of 97 per cent. Methyl ricinoleate of high purity can be prepared more readily and simply than the acid,[69], [70] but saponification of the ester to obtain the pure acid is unsatisfactory.

Castor oil is of considerable commercial importance since its component ricinoleic glycerides can be dehydrated readily to a mixture of conjugated and non-conjugated linoleic acid glycerides, useful in surface coating applications.[71] Reaction with aqueous alkali at high temperatures and preferably under pressure converts castor oil or esters of ricinoleic acid, as well as the free acid, to sebacic acid, an important component of plastics, plasticizers, and synthetic lubricants.[72], [73] 2-Octanol is also formed in this reaction. Pyrolysis of ricinoleic acid yields undecylenic acid and heptaldehyde. Ricinelaidic acid, the *trans* isomer, melting point, 52° and $[\alpha]_D^{20}$ + 6·67° (ethanol), is readily prepared from ricinoleic acid by reaction with oxides of nitrogen. This isomer can also be prepared directly from castor oil by elaidinization and hydrolysis. Ricinelaidic acid does not occur naturally.

IV. DIHYDROXY ACIDS

Dihydroxy fatty acids contain two hydroxyl groups in the fatty acid chain. Only saturated dihydroxy acids will be discussed here and the emphasis will be on those containing eighteen carbon atoms. The hydroxyl groups may be on adjacent (contiguous) or non-adjacent carbon atoms. The former (α-glycol) type are more important by far because reactions for their preparation have been well developed.

Dihydroxy fatty acids are uncommon in nature. The most important one is the optically active form of high-melting 9,10-dihydroxystearic acid, melting point 141°, which has been isolated in small amounts from the mixed fatty acids of castor oil[74] and, presumably, also from lycopodium oil.[75] 3,11-Dihydroxytetradecanoic (purolic) acid has been reported as a component acid of the seed fats of the South African and Japanese morning glories.[76], [77] A dihydroxy acid (lanoceric acid), $C_{30}H_{60}O_4$, has apparently been isolated from wool wax.[78], [79]

(*a*) *Preparation*

Two general and widely used methods are available for the preparation of dihydroxy fatty compounds of the α-glycol type. These are epoxidation of the corresponding olefinic acid with organic peracids followed by hydrolysis,[1], [2] and oxidation of the olefinic acid with dilute cold alkaline potassium permanganate.[63] Yields of dihydroxy acid are good and frequently exceed 90 per cent.

Since each dihydroxy acid (with the exception of those with a terminal hydroxyl) contains two asymmetric carbon atoms, there are four optical

isomers possible or two racemic mixtures. As a rule the dihydroxy acid obtained by peracid oxidation of the *cis* form of the olefinic acid is the low-melting racemic mixture, and that obtained by potassium permanganate oxidation is the high-melting racemic mixture.[18], [19] As discussed earlier, epoxidation with organic peracids proceeds by *cis*-addition, but hydrolysis to the dihydroxy acid involves an inversion. On the other hand, potassium permanganate reacts by *cis* or normal addition. It is possible, therefore, to prepare the same racemic mixture of dihydroxy acids from the *cis* or *trans* form of the olefinic acid by oxidizing one with organic peracids and the other with alkaline potassium permanaganate.

In the low-melting racemic mixture (for example, 9,10-dihydroxystearic acid, melting point 95°, prepared from oleic acid by peracetic acid oxidation and hydrolysis, or from elaidic acid by hydroxylation with alkaline potassium permanganate) the hydroxyl groups are closer together in space than in the high-melting racemic mixture (9,10-dihydroxystearic acid, melting point 131°, prepared from oleic acid by hydroxylation with alkaline potassium permanganate, or from elaidic acid by peracetic acid oxidation and hydrolysis). This was recently shown to be the case in an unambiguous manner on the basis of the ability of the low-melting racemic mixture to form a urea complex readily whereas the high-melting racemic mixture did not.[19] This conclusion is in accord with chemical evidence, namely, the comparative rates of cleavage of the isomeric 9,10-dihydroxystearic acids with periodic acid and lead tetraacetate.[80], [81]

Other useful procedures for preparing contiguously substituted dihydroxy fatty acids are addition of halogen or hypochlorous acid to the olefinic acid followed by hydrolysis with aqueous or alcoholic alkali or silver oxide,[7] reaction of olefinic acids with silver iodobenzoate (Prévost's reaction) followed by hydrolysis,[82], [83], [84] oxidation of olefinic acids with *tert*-butyl hydroperoxide or hydrogen peroxide in *tert*-butyl alcohol in the presence of metal oxides, such as osmium tetroxide, vanadium pentoxide and chromium trioxide, which are capable of forming inorganic peracids,[85], [86], [87] and photochemical addition of hydrogen peroxide to the double bond.[88] The Prévost reaction proceeds by *trans* addition to the double bond, whereas the photochemical or metal-catalyzed addition of hydrogen peroxide or *tert*-butyl hydroperoxide proceeds by *cis* addition.

Fig. 1 correlates the stereochemical relationships involved in the conversion of oleic and elaidic acids to the 9,10-dihydroxystearic acids by way of the intermediate oxirane and chlorohydroxy compounds.[18] This scheme is self-consistent and is in harmony with accepted theories of the Walden inversion and double bond addition reactions. According to this scheme, epoxidation of the unsaturated acids with organic peracids, or oxidation with alkaline potassium permanganate, takes place by *cis* addition, whereas reaction with hypochlorous acid proceeds by *trans* addition. Furthermore, opening (either in alkaline or acidic media) and reforming of the oxirane ring, as well as replacement of a hydroxyl group of 9,10-dihydroxystearic acid with halogen, are each

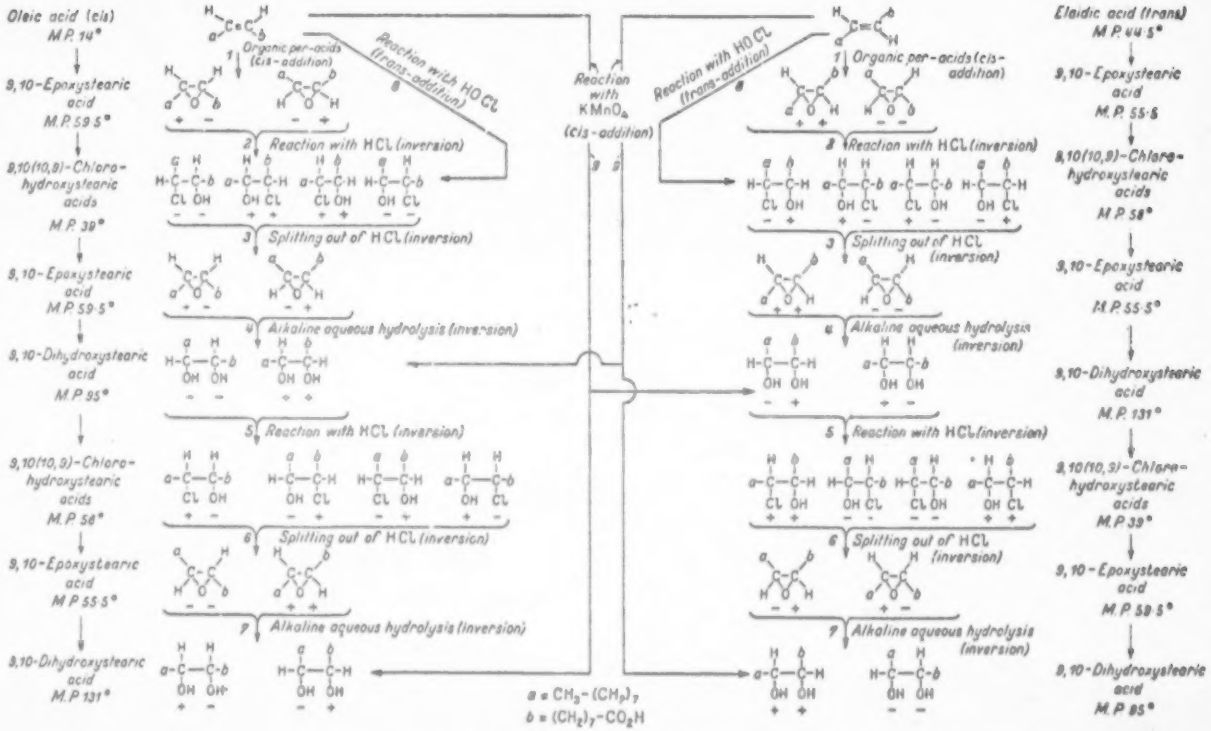


Fig. 1. Stereochemical relationships in the conversion of oleic and elaidic acids to the 9,10-dihydroxystearic acids

accompanied by an inversion. An inversion also occurs when halogen is replaced by hydroxyl in the 9,10 (10,9)-halohydroxystearic acids. This scheme is generally applicable to olefinic acids, esters and alcohols and is now accepted by the majority of investigators in the field.[11]

Dihydroxy fatty acids in which the hydroxyl groups are non-contiguous are not well known. Reaction of ricinoleic acid with sulphuric acid followed by hydrolysis yields a dihydroxystearic acid, but the position of the hydroxyl groups is not known.[89] Reaction of linoleic acid with sulphuric acid gives a low yield of a dihydroxystearic acid of unknown structure and some monohydroxyoleic acid (by addition of sulphuric acid to only one of the double bonds in linoleic acid), as well as oxygen-linked polymers.[44] Formic acid adds to linoleic acid and, after hydrolysis, gives a good yield of a mixture of approximately equal parts of dihydroxystearic acid and monohydroxyoleic acid.[47] The structure of these compounds has not yet been reported. Reaction of formaldehyde with monoolefinic acids (Prins' reaction) in the presence of sulphuric acid yields a complex mixture of products[90, 91] from which it has been claimed that cyclic dihydroxy compounds can be isolated.[90] This conclusion has been questioned.[51]

(*b*) *Reactions and properties*

Dihydroxy fatty acids are white, high-melting crystalline solids. The longer-chain members are insoluble in water whereas the shorter chain ones are either slightly or completely soluble (less than ten carbon atoms). They are soluble in hot oxygenated organic solvents, such as ethanol and acetone, but insoluble in hydrocarbons.

Dihydroxy fatty acids show both hydroxyl and carboxyl reactivity. However, esters or ethers in which only one hydroxyl group is bound up are best prepared by the reaction of the corresponding oxirane with an acid or alcohol, respectively. Reaction of the second hydroxyl group is somewhat more difficult to accomplish because of steric hindrance and/or electronic effects.[92, 93]

Thermal treatment of contiguously substituted dihydroxy acids yields a variety of products depending on reaction time, temperature and catalysts used. For example, 9,10-dihydroxystearic acid has been reported to yield epoxystearic acid,[94, 95] estolides,[96] 10-ketostearic acid,[95, 97] hydroxyolefinic acids[95] and diunsaturated acids.[95] In the most recent report,[95] 9,10-dihydroxystearic or its methyl ester has been shown to yield 43 to 47 per cent conjugated diunsaturated acids, 31 to 36 per cent non-conjugated diunsaturated acids, 5 to 11 per cent keto acids, 7 to 8 per cent epoxy acids and 4 to 7 per cent hydroxyolefinic acid when heated to 290° with 0·5 per cent of toluenesulphonic acid for 3 minutes at 200 mm. Thermal polymerization of dihydroxy fatty acids yields polyesters, with the elimination of water. Since the acid molecules are trifunctional, cross-linking and gelation occur readily. Esters of the dihydroxy acids are bifunctional, however, and may be employed for the preparation of linear polymers by reaction with dibasic acids.[98]

Contiguously substituted dihydroxy acids undergo a complicated reaction

when heated with aqueous alkali at high temperatures.[99], [100], [101], [102] Presumably, they first dehydrogenate to the diketo acid which then undergoes a benzilic acid rearrangement.[102] Thus, 9,10-dihydroxystearic acid is reported to yield 2-octyl-2-hydroxydecanedioic acid, and 9,10-dihydroxybehenic acid yields 2-octyl-2-hydroxytetradecanedioic acid.[100] The presence of potassium chlorate in the fusion reaction causes the aliphatic chain to cleave yielding mono- and dibasic acids.[103]

Contiguously substituted dihydroxy fatty acids are readily cleaved to aldehydes with lead tetraacetate,[80], [104] periodic acid,[81] sodium bismuthate in acid medium,[105] and manganese tri-[106] or tetraacetate.[107]

The 9,10-dihydroxystearic acids are the best known members of this class of compounds. The 2,3-dihydroxystearic acids[108] and those with the hydroxyl groups in the 6,7 through the 12,13-position have recently been studied in some detail.[109], [110], [111] Both the low-melting and the high-melting series of 6,7- through 12,13-dihydroxystearic acids (obtainable, respectively, from the *cis* and *trans* series of octadecenoic acids by performic acid oxidation) show alternation of properties. By melting point and interplanar spacings, these acids can be classified into four sub-groups, namely, the even (6,7-, 8,9-, etc.) and the odd (7,8-, 9,10-, etc.) low-melting and the even and odd high-melting isomers. Within each sub-group the individual members cannot be satisfactorily distinguished by melting points and interplanar spacings. They can be distinguished, however, by the relative intensities of the different orders of their long spacings. The composition of mixtures of low- (95°) and high-melting (131°) 9,10-dihydroxystearic acids can be determined to within $\pm$ 3 per cent from the relative intensity of a selected pair of diffraction lines obtained in the x-ray powder patterns of such mixtures.[22]

Table 2 lists the better known contiguously substituted dihydroxyoctadecanoic acids.

Table 2. Contiguously substituted dihydroxyoctadecanoic acids

Hydroxyl positions	*Low-melting Racemic mixtures*[a] *M.P., °C*	*High-melting Racemic mixtures*[b] *M.P., °C*
2,3-[c] . . .	123–124°	106–107°
6,7- . . .	119°	124°
7,8- . . .	95–96°	132–133°
8,9- . . .	96–97°	118–119°
9,10- . . .	95°	130–131°
10,11- . . .	98–99°	120–121°
11,12- . . .	95–96°	129–130°
12,13- . . .	98·5°	119–120°

[a] Prepared from the *cis*-forms of the corresponding octadecenoic acids by oxidation with performic acid.
[b] Prepared from the *trans*-forms of the corresponding octadecenoic acids by oxidation with performic acid.
[c] The 2,3-dihydroxystearic acids appear to be the exceptions to the rule that the low-melting racemic mixture is obtained by peracid oxidation of the *cis*-form of the octadecenoic acid.

V. Polyhydroxy Acids

Preparation

The best known hydroxy fatty acids containing more than two hydroxyl groups in the fatty acid chain are those prepared from ricinoleic, linoleic and linolenic acids, and contain, respectively, three, four and six hydroxyl groups per molecule.

Hydroxylation of ricinoleic and ricinelaidic acids with alkaline potassium permanganate and peracetic acid yields four diastereoisomeric 9,10,12-trihydroxystearic acids formed in two inter-related pairs, as expected from theoretical considerations.[112] The α- and β-acids, melting point 112° and 138°, are obtained, respectively, from ricinoleic acid by hydroxylation with alkaline potassium permanganate and from ricinelaidic acid by reaction with peracetic acid followed by hydrolysis. The γ- and δ-acids, melting point 87° and 110°, are similarly prepared from ricinelaidic and ricinoleic acids by hydroxylation with alkaline potassium permanganate and peracetic acid, respectively.

Hydroxylation of octadecadienoic acids yields tetrahydroxystearic acids containing four asymmetric carbon atoms. Therefore, eight racemic mixtures and sixteen optically active forms should exist. The eight racemic mixtures of 9,10,12,13-tetrahydroxystearic acid have been prepared;[113] these are called sativic acids. The spatial arrangements of the hydroxyl groups in each diastereoisomer have been deduced.[113]

Hydroxylation of linoleic acid with alkaline potassium permanganate[114] yields two racemic forms, melting points 174° and 164°. Two additional racemic forms, melting points, 146° and 126° or 122°, are obtained from linoleic acid by treatment with hypochlorous and hypobromous acid followed by hydrolysis,[115] by epoxidation of linoleic acid with peracetic acid followed by hydrolysis,[83] or by hydroxylation of linolelaidic acid with alkaline potassium permanganate.[116] A third pair, melting points, 164° and 126° is prepared by the oxidation of the *trans-cis* or *cis-trans* geometric isomers of linoleic acid with alkaline potassium permanganate.[116] The fourth pair, melting points 164° and 141·5°, is prepared from 9,10-dihydroxy-*cis*-12-octadecenoic acid by hydroxylation with alkaline potassium permanganate.[113] The tetrahydroxystearic acids are slightly soluble in boiling water.

Hydroxylation of octadecatrienoic acids yields hexahydroxystearic acids containing six asymmetric carbon atoms. Of the thirty-two possible racemic mixtures (sixty-four optically active forms), only two have been adequately characterized.[117] These are linusic and isolinusic acids, melting points 203° and 173·5°, respectively, prepared in low yield by oxidation of linolenic acid with alkaline potassium permanganate. No information on the spatial arrangement of the hydroxyl groups in these acids has been reported, although it is possible to make certain deductions on the basis of *cis*-hydroxylation. The hexahydroxystearic acids are soluble in boiling water.

VI. Keto Acids

(a) *Preparation*

Keto fatty acids are straight-chain aliphatic monocarboxylic acids containing one or more carbonyl groups in the fatty acid chain. They may be saturated or unsaturated.

Only a few keto acids are naturally occurring. α-Licanic acid, 4-keto-9,11,13-octadecatrienoic acid, melting point 74°, is the major component acid (80 per cent) of the glycerides of oiticica oil. It was first described by Wilborn[118] who isolated it from the seed fat of *Couepia grandiflora*, but its major source is oiticica oil obtained from the seed fat of *Licania rigida*.[118] Mexican oiticica oil from *Licania arborea*[120] and po-yoak oil from *Parinarium sherbroense*[121] are also good sources. Its structure was demonstrated by Brown and Farmer[119] who showed that its double bonds are conjugated and that it yields 4-keto-stearic acid on hydrogenation. Isomerization of α-licanic acid with iodine or sulphur, or exposure to sunlight, yields β-licanic acid,[119] melting point, 97°.

Lactorinic acid, 6-ketostearic acid, melting point 86 to 87°, has been isolated in 1 to 3 per cent yield from the fat of the fungus *Lactarius rufus*,[122], [123] and small amounts of keto acids are reported to be formed by the action of micro-organisms on fats.[31]

Most of the known keto acids are synthetic substances. The most frequently used synthetic methods are hydration of the corresponding acetylenic acids, condensation reactions involving β-keto esters (malonates or acetoacetates) and condensations using organo-metallic compounds (magnesium, zinc or cadmium).

Hydration of the corresponding acetylenic acids in the presence of strong acids, such as formic and sulphuric, or by direct addition of water under pressure at high temperatures,[31], [121], [125] are the classical methods for preparing keto fatty acids. Hydration of acetylenic acids has also been accomplished with alcoholic potassium hydroxide,[126] and by hydrolysis of the addition products of mercuric salts in acid media.[127] A mixture of two isomeric monoketo acids, which usually can be separated, is obtained by these reactions. Thus, hydration of stearolic acid, 9-octadecynoic acid, yields a mixture of 10-keto- and 9-ketostearic acid, the former predominating, and this ratio is reported to increase as the triple bond moves closer to the carboxyl group.[125]

The development of methods for the synthesis of short-chain acetylenic compounds within the past decade, coupled with their commercial availability, has made the preparation of monoketo acids from acetylenic acids of considerably greater practical significance than formerly. Acetylenic acids can be readily prepared[109], [111], [126], [127], [127a], [127b], [128] by condensing an alkylacetylene with an α,ω-chloroiodoalkane to yield a 1-chloroalkyne which is then converted to the nitrile and hydrolyzed to the alkynoic acid, as illustrated:

$$CH_3—(CH_2)_m—C{\equiv}CH + I(CH_2)_nCl \xrightarrow{NaNH_2} CH_3(CH_2)_m—C{\equiv}C—(CH_2)_nCl$$

$$CH_3(CH_2)_mC{\equiv}C—(CH_2)_nCl \xrightarrow[\substack{2.\ NaOH\\3.\ Acid}]{1.\ NaCN} CH_3—(CH_2)_m—C{\equiv}C—(CH_2)_nCOOH$$

Another convenient method for preparing acetylenic acids, especially stearolic, is from the corresponding olefinic acid by bromination and dehydrobromination with alcoholic potassium hydroxide[129] or, preferably, sodium amide in liquid ammonia.[130]

Another important and widely used synthesis of monoketo acids is the condensation of carbethoxy acyl halides with the sodium derivative of either ethyl *n*-alkyl malonates or, preferably, ethyl 2-acetylalkanoates (alkyl acetoacetates) followed by cautious hydrolysis.[31], [131] Yields of monoketo acids are fair, but improved yields can be obtained by condensing the sodium derivative of ethyl acetoacetate with ω-bromo esters and subsequent reaction with acyl chlorides followed by hydrolysis,[132] as illustrated—

$$CH_3COCH(Na)COOC_2H_5 + Br(CH_2)_nCOOC_2H_5$$

$$\downarrow$$

$$CH_3COCH(COOC_2H_5)(CH_2)_nCOOC_2H_5$$

$$\downarrow \; Na,\ CH_3(CH_2)_mCOCl$$

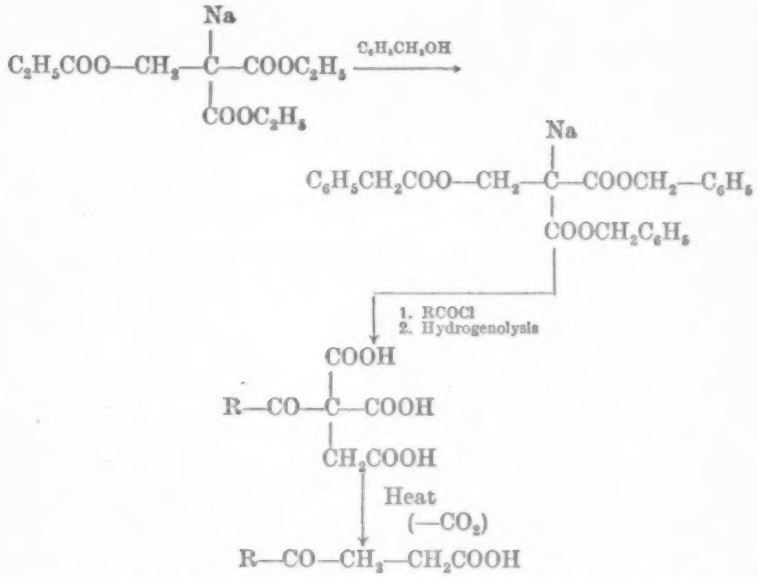
$$CH_3COC(COOC_2H_5)(CH_2)_nCOOC_2H_5$$

$$\quad\quad\quad

contiguously substituted diketo acids from the corresponding dihydroxy acids.[111]

Synthetic methods which are specific for the synthesis of 2- and 3-keto acids are also known.[50], [51], [142], [143], [144]. Some of these are the Reformatsky reaction and hydrolysis of α-ketonitriles or α-oximino esters. The Reformatsky reaction is especially useful.

4-Keto acids can be prepared by a synthesis which takes advantage of the facile hydrogenolysis of benzyl esters.[145] In this sequence of reactions, illustrated below, the sodium derivative of triethyl ethane-1, 1,2-tricarboxylate is converted to its tribenzyl derivative which is then condensed with an acyl chloride. Catalytic debenzylation followed by decarboxylation yields the keto acid:



Other applications and modifications of this debenzylation synthesis have been published.[146], [147], [148], [149]

In an interesting synthetic method recently reported, it was shown that C_6 through C_{11} 5-keto aliphatic acids (or nitriles) can be prepared in fair yields by the reaction of β-ketoesters with acrylonitrile followed by hydrolysis:[150]

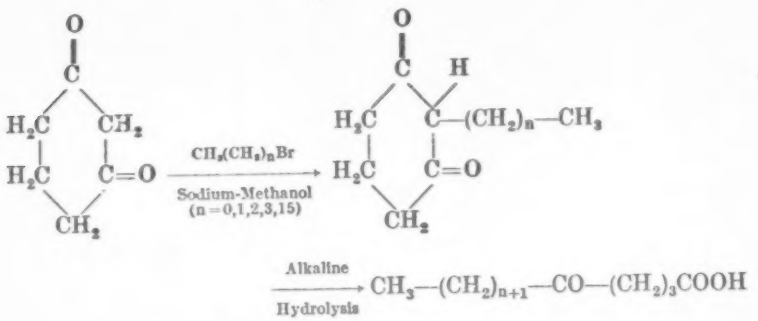
$$RCOCH_2COOC_2H_5 + CH_2{=}CHCN \xrightarrow[\text{Ethanol}]{\text{Na}} RCOCH(CH_2CH_2CN)COOC_2H_5$$

(R=CH_3, C_2H_7, C_4H_9, C_5H_{11}, C_6H_{13}, etc.)

$$\xrightarrow[\text{Alkaline Hydrolysis}]{\text{Acid or}} RCO(CH_2)_3CN \text{ or } RCO(CH_2)_3COOH$$

The method appears applicable to longer-chain homologues.

Another interesting synthetic method for preparing 5-keto aliphatic acids in good yields involves C-alkylation of 1,3-cyclohexanedione, which exists predominantly as an enol, followed by alkaline hydrolysis:[151]



This procedure should be applicable to the preparation of the entire homologous series. It has also been used to prepare phenyl substituted and unsaturated keto acids, as well as dibasic acids.[152]

Several of the synthetic methods described, or modifications of them, have recently been employed to prepare 2- through 17- keto-octadecanoic acids and their methyl esters.[43] As mentioned earlier, thermal and x-ray data have also been reported, thereby permitting unequivocal identification of any isomer.

(*b*) *Reactions and properties*

Keto fatty acids are white crystalline solids insoluble in water but soluble in hot organic solvents. They have typical ketone properties and form the usual derivatives with phenylhydrazine, hydroxylamine and semicarbazide. In general, keto acids are stable, distillable compounds but 2-keto acids lose carbon monoxide on heating and yield the next lower saturated acid.[153] Heating with aqueous sulphuric acid causes 2-keto acids to lose carbon dioxide yielding aldehydes containing one less carbon atom than the acids from which they are derived.[154] 3-Keto acids may also lose carbon dioxide readily to yield a ketone. Keto acids can be reduced to the corresponding hydroxy acids by catalytic hydrogenation or to saturated acids by the Clemmensen reduction.[31], [155]

Monoketo fatty acids are useful in unequivocally proving the structure of monohydroxy and unsaturated acids. Hydroxy acids can be readily converted to the corresponding keto acids in high yields. These are then converted to the oxime followed by Beckmann rearrangement, hydrolysis and identification of the shorter-chain fragments.[156] The position of the double bond in oleic acid was first shown to be in the 9-position by conversion to stearolic acid and hydration to a mixture of 9- and 10-ketostearic acids, followed by the Beckmann rearrangement procedure just outlined.[157] Simpler techniques are now available for proving the position of double bonds and the keto acid-oxime procedure is infrequently employed.

Table 3. Saturated monoketo fatty acids[a]

Systematic name	*M.P., °C*	*Derivative*
2-Ketododecanoic	56·5–57°	
3-Ketododecanoic		Ethyl ester, b.p. 164–165°/13
9-Ketododecanoic	56°	Semicarbazone, m.p. 131°
10-Ketododecanoic	72°	
2-Ketotridecanoic	62–62·5°	
10-Ketotridecanoic	63°	
12-Ketotridecanoic	70–71°	B.p., 185–186°/1
2-Ketotetradecanoic	63·8°	
3-Ketotetradecanoic	39–40°	Semicarbazone, m.p. 253°
4-Ketotetradecanoic	87°	Oxime, m.p. 74°
10-Ketotetradecanoic	69°	
11-Ketotetradecanoic	66–67°	Semicarbazone, m.p. 100°
13-Ketotetradecanoic		Ethyl ester, m.p. 28–29°; b.p. 164–166°/1
12-Methyl-10-ketotridecanoic	54–55°	
12-Methyl-9-ketotridecanoic	50·5–51°	
2-Methyl-12-ketotridecanoic	46·5–47·2°	Methyl ester, b.p. 181–183°/11
2-Ketopentadecanoic	68–68·5°	
4-Ketopentadecanoic	92·6°	
11-Ketopentadecanoic	70–71°	
14-Ketopentadecanoic	78·4–79·4°	Methyl ester, m.p. 43·2–43·8°; b.p. 205–206°/15
2-Ketohexadecanoic	69°	
4-Ketohexadecanoic	91–92°	Oxime, m.p. 54°
5-Ketohexadecanoic	88°	
7-Ketohexadecanoic	78°	
8-Ketohexadecanoic	77–78°	
9-Ketohexadecanoic	73·5–74·5°	
10-Ketohexadecanoic	75–75·8°	
11-Ketohexadecanoic	74–75°	
14-Methyl-10-ketopentadecanoic	68–69°	
9-Ketoheptadecanoic	78·5°	Semicarbazone, m.p. 111°; amide m.p. 119°
2-Ketooctadecanoic	74·5°	Methyl ester, m.p. 54°
3-Ketooctadecanoic	99° (dec.)	Semicarbazone, m.p. 295–300°; methyl ester, mp. 49°
4-Ketooctadecanoic	97°	Semicarbazone, m.p. 126°; oxime, m.p. 85°; methyl ester, m.p. 48°
5-Ketooctadecanoic	87°; 91°	Methyl ester, m.p. 54·5°
6-Ketooctadecanoic	87°	Oxime, m.p. 59–61°; methyl ester, m.p. 46·5°
7-Ketooctadecanoic	83°	Methyl ester, m.p. 49°
8-Ketooctadecanoic	84°	Methyl ester, m.p. 46·5°
9-Ketooctadecanoic	83°	Semicarbazone, m.p. 118–120°; methyl ester, m.p. 47·5°
10-Ketooctadecanoic	82·5°	Semicarbazone, m.p. 100–101°; methyl ester, m.p. 46°; ethyl ester, m.p. 41°

Table 3—continued

Systematic name	*M.P., °C*	*Derivative*
11-Ketooctadecanoic	82°	Methyl ester, m.p. 47°
12-Ketooctadecanoic	81·5°	Semicarbazone, m.p. 125°; methyl ester, 45°
13-Ketooctadecanoic	82°	Methyl ester, m.p. 47·5°
14-Ketooctadecanoic	82°	Methyl ester, m.p. 49°
15-Ketooctadecanoic	83°	Methyl ester, m.p. 53·5°
16-Ketooctadecanoic	93°	Methyl ester, m.p. 54·5°
17-Ketooctadecanoic	87·5°	Methyl ester, m.p. 56°
16-Methyl-10-ketoheptadecanoic	71·2–72°	
10-Ketononadecanoic	86–87°	
13-Ketoheneicosanoic	89–90°	Semicarbazone, m.p. 104–105°
4-Ketodocosanoic	103°	
10-Ketodocosanoic	94°	
14-Ketodocosanoic	84·5°	Oxime, m.p. 49–51°
10-Ketotricosanoic	93·5°	
10-Ketotetracosanoic	94–94·5°	
13-Ketotetracosanoic	95°	
13-Ketotriacontanoic	104°	
18-Ketononatriacontanoic	110·3–110·6°	
13-Ketodotetracontanoic	110°	

[c] The data in this table have been compiled from the same reference works listed in footnote [c] of Table 1, the literature references given in the section of this chapter entitled "Keto Fatty Acids," and unpublished research carried out in the author's laboratory.

Alkyl esters of 12-ketostearic acid have been patented as plasticizers and stabilizers for gums and resins.[158]

Table 3 lists the better known saturated monoketo fatty acids containing twelve or more carbon atoms.

VII. Miscellaneous Oxygenated Fatty Acids

(*a*) *Hydroxyketo acids*

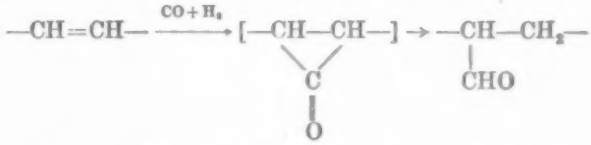
9 (10), 10 (9)-Hydroxyketostearic acids, apparently formed in equal amounts, have been prepared by oxidizing oleic and elaidic acids at low temperatures with neutral, dilute, aqueous potassium permanganate solution.[159], [160], [161] Excess alkali must be avoided[160] otherwise an almost theoretical yield of high-melting, 9,10-dihydroxystearic acid, m.p. 131°, is obtained from oleic acid, and low-melting 9,10-dihydroxystearic acid, m.p. 95°, from elaidic acid.[162] The isomeric hydroxyketostearic acids have been separated by fractional crystallization of their semicarbazones, followed by hydrolysis.[160] 9-Hydroxy-10-ketostearic acid melts at 74°; 10-hydroxy-9-ketostearic acid

melts at 75·5°. Both isomers are readily soluble in most organic solvents. The mechanism of their formation from oleic acid is not known; 9,10-dihydroxystearic acid is not an intermediate.

The hydroxyketostearic acids are cleaved with periodic acid at room temperature and with acidic potassium permanganate at elevated temperatures.[160] Mild chromic acid oxidation yields diketostearic acid, and reduction yields 9,10-dihydroxystearic acid, melting point 131°.[160] Fehling's solution and ammoniacal copper solutions are also reduced by hydroxyketostearic acids.[160] Of interest also is the facile cleavage of these acids in good yield in alkaline solution with gaseous oxygen to pelargonic and azelaic acids.[160], [161], [163] The cleavage of the chain is dependent mainly on the excess of alkali present.[161] In alkaline solution each of the hydroxyketostearic acids is converted to the equilibrium mixture in 24 to 36 hours at room temperature or within a few minutes at 100°.[160]

(*b*) *Oxo reaction*

Reaction of olefins under pressure with carbon monoxide and hydrogen in the presence of certain metallic catalysts yields aldehydes, as shown:



These are usually reduced further to the corresponding alcohols, or they can be oxidized to acids.[49], [164], [165] If a long-chain olefin, such as methyl oleate, is the starting material one can prepare branched aldehydo acids, hydroxy acids or dibasic acids by means of this reaction. Although the oxo reaction has considerable promise, it has not received extensive study with long-chain olefinic materials derivable from fats.

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ADDENDUM (APRIL, 1954)

IN the preparation of this chapter, the literature was consulted through 1952. Since then this field has been remarkably active and about fifty pertinent literature references have appeared. These are listed below in groups which correspond to the main subdivisions within the chapter.

Of interest are the papers by JUNGERMANN and SPOERRI and by MACK and BICKFORD who showed that the reaction of 9,10-epoxystearic acid with hydrogen chloride or hydrogen gave predominantly 10-hydroxy-9-chlorostearic acid and 10-hydroxystearic acid, respectively.

CHIBNALL, PIPER and WILLIAMS have shown conclusively that phrenosinic (cerebronic) acid is a mixture of 85 per cent α-hydroxytetracosanoic and 15 per cent α-hydroxyhexacosanoic acid. HORN, HOUGEN and VON RUDLOFF have shown that α-hydroxy acids containing 12, 14, 16, and 18 carbon atoms comprise nearly 30 per cent of the acids of wool wax, with the C_{16} predominating. An unusual hydroxy acid, heptadec-10-en-8-yne-7-hydroxy-1-carboxylic acid, has been isolated by Ligthelm from the oil of *Ximenia caffra*.

By determination of the optical activity of the methyl esters and the acetyl derivatives of castor oil acids, BOLLEY has shown that castor oil contains 90–93 per cent of ricinoleic acid (as the glyceride). KNAFO has prepared pure ricinoleic acid from the mixed fatty acids of castor oil by acetylation of the hydroxy acids and precipitation of the straight-chain acids as urea complexes, followed by isolation of ricinoleic acid from the non-complexed acetates.

AEBI, VILKAS and LEDERER and ASSELINEAU have shown that α-mycolic acid is a mixture of a dihydroxy acid, $C_{87}H_{174}O_4 \pm 5\,CH_2$, with a hydroxy and methoxy acid. A provisional structure is reported for the dihydroxy acid. LOGAN has shown that 9,10-dihydroxystearic acid can be cleaved to azelaic and pelargonic acids in high yield by fusion with aqueous alkali at elevated temperatures. MCGHIE has prepared ketohydroxy acids from 9,10-dihydroxystearic and 13,14-dihydroxybehenic acids by oxidation with chromic acid to the corresponding diketo acids, followed by partial reduction with zinc.

Improved syntheses of ketones and keto acids have been described by Bowman and Fordham; Buu-Hoï; Kreuchunas; Lettré and Jahn; Manyik, Frosteck, Sanderson and Hauser; Smuszkowicz; Stetter and Klanke; Stetter and Dierichs; Viscontini; and Yoho and Levine.

Gunstone has reviewed recent developments in the preparation of natural and synthetic fatty acids.

(*a*) *Epoxy Acids*

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LOW PRESSURE FRACTIONAL DISTILLATION AND ITS USE IN THE INVESTIGATION OF LIPIDS

K. E. Murray

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7

LOW PRESSURE FRACTIONAL DISTILLATION AND ITS USE IN THE INVESTIGATION OF LIPIDS

*K. E. Murray**

I. Introduction

Fractional distillation at low pressure has for long been a powerful and virtually indispensable tool in lipid chemistry, its main field of application being to the analysis and isolation of the constituent fatty acids, where complex mixtures are often encountered.

The technique of laboratory fractional distillation made great advances in the 'thirties to meet the needs of research on the constituents of petroleum. The efficient fractionating columns so developed for use at atmospheric pressure were soon adapted to the low pressure distillation of the higher fatty acid esters, and the much improved separations which they gave made possible the development of the analytical procedures now in use for fatty acid mixtures. However, operation at low pressures introduces special problems, and appreciation of these has led in recent years to the development of efficient equipment specially designed for low pressure work. With the accumulation of reliable data for the vapour pressures of fatty acid esters, and the introduction of the method of amplified distillation, such equipment has made the fractionation of fatty acids a procedure of high efficiency and wide applicability.

This article is an attempt to survey the recent practice of laboratory low pressure batch distillation and its application to the fractionation of fatty-acid esters, but the techniques may well be applied to other substances of comparable molecular size. Because of the great volume of the literature an exhaustive review has not been attempted, but it is believed that the more important contributions to fatty acid ester distillation have been covered, especially those since the reviews of Markley (1947) and Ralston (1948). For a full general account of laboratory distillation and its terminology reference should be made to the recent works of Carney (1949) and Weissberger (1951). The subject has been critically reviewed by Given (1950). Classified abstracts of the literature on distillation for the years 1941–45 and 1946–52 have been published by Rose and Rose (1948, 1953).

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II. Low Pressure* Fractional Distillation

A. Some factors of special importance in low pressure distillation

Owing to the hazard of thermally decomposing the less stable and longer chain fatty compounds, it is naturally desirable to operate at as low a pressure as possible: in the range 1 to 10 mm halving the pressure lowers the boiling point 13 to 16°C. When fractional distillation is performed at low pressures, several factors having negligible effect at atmospheric pressure become of importance, and may make an apparatus well suited to use at normal pressures both difficult to operate and of reduced efficiency. For example, the pressure drop through the column becomes greater in relation to the total pressure, the charge may evaporate in a troublesome manner, and the high vapour velocity and other factors may reduce the fractionating efficiency of the column.

1. Pressure drop

The pressure drop through a column is a characteristic of the type of packing or column and a function of the vapour velocity or boil-up rate. When a column is operated at atmospheric pressure the pressure drop is small in relation to the total pressure, but if the head pressure is reduced the pressure drop, which does not alter greatly, becomes larger in relation to the head pressure, and eventually may become many times its value. In the attempt to reduce the thermal hazard of material in the still pot to a minimum many investigators perform distillations in packed columns at a head pressure, which because of a large pressure drop, is only a small fraction of that in the still pot, and temperature differences of up to 100°C between column head and still pot are frequently reported. Norris and Terry (1945) have shown that such operation does not achieve the desired purpose. They distilled methyl palmitate through a column of large pressure drop (26 mm at 1·2 mm) and noted an increase of 1°C when the head pressure was raised from 1 to 2 mm and only 7°C for an increase from 1·2 to 10 mm. Furthermore it has been observed that with a spinning band column (Murray, 1951) of small pressure drop (0·5 mm at 0·5 mm) that little decrease in the temperature of the charge resulted from working at head pressures below 0·5 mm.

Since the main purpose of distilling at reduced pressures is to lower the temperature of the still-pot contents to avoid or reduce decomposition, it is obvious from the above that the pressure drop characteristic of the column determines the lowest pressure at which it may be effectively used, and this pressure appears to correspond roughly to the pressure drop. However, as the latter varies considerably with boil-up rate at low pressures, operation is possible at lower pressures by reducing the boil-up rate. The restriction placed on packed columns generally by their relatively large pressure drop has led

* The term "low pressure distillation" is preferred to "vacuum distillation" as being less vague and quite distinct from "high vacuum distillation" which usually signifies molecular distillation. For the purpose of this review it is taken to cover the pressure range 0·1 to 10 mm, all pressures referred to being in millimetres of mercury.

investigators to develop columns which combine high efficiency and a small pressure drop, particularly for use at low pressures.

2. The evaporative behaviour of the distilland in the still pot

Uneven boiling or "bumping" of the distilland can make efficient fractional distillation at low pressures impracticable, and many devices to cause more even boiling have been suggested with varying degrees of success (see Bowman and Tipson, 1951). Until recently little had been done to study the conditions of vaporization at low pressures, but now two factors may be recognized in relation to efficient column operation. These are, (*a*) the cause of uneven evaporation and associated superheating of the charge and (*b*) the effect of stirring the charge on the efficiency of fractionation.

(*a*) Distillation at pressures lower than about 2 mm, in the absence of decomposition, takes place from a quiet surface as an evaporative process, and under these conditions a considerable degree of superheating may occur which can lead to explosive boiling (Birch, Gripp, and Nathan, 1947). In a deep, unstirred charge heated externally from below, ebullition is prevented in the lower layers by the hydrostatic head of liquid being greater than the vapour pressure. The heat required for evaporation from the surface must therefore be conducted through the charge and a large temperature gradient between wall and surface will result. If a temperature high enough to overcome the effect of the hydrostatic head is reached, the superheating will cause rapid or explosive boiling. When this ceases the process will be repeated. The combined recent studies of Hickman and Trevoy (1952) have shown that this picture is far from complete. They have recognized that evaporation is governed by surface conditions, and that liquids tend to form a surface film which hinders evaporation. The film is probably due to traces of a soluble impurity preferentially located in the surface of any undisturbed liquid. The effect is most pronounced in the range of 0·001 to 10 mm projective pressure when the surface may separate into two clearly distinguishable areas, a smooth "torpid" section and a violently agitated "active" or "working" section. Certain mixed liquids present an entirely torpid surface, some chemically pure liquids an all-active surface, but the usual condition is "schizoid" with both present (Plate I). The rate of evaporation from the torpid area may be much less than that from the working area by a factor of one-half to one-thousandth, and because of this considerable superheating in the torpid surface may occur. Once formed the torpid area tends to dominate and engulf the working area, and a rise in temperature of 10° to 15° may be necessary to puncture the torpid surface and re-establish working conditions. The resulting explosion of vapour cools the liquid and torpidity again becomes uppermost.

(*b*) Birch, Gripp, and Nathan (1947) have observed a pronounced improvement in the fractionating efficiency of their spinning band column when the charge was stirred. This is shown in a striking manner in Fig. 1 by the comparative fractional distillations of hydrogenated rape-seed oil fatty acid methyl esters performed with and without mechanical stirring of the charge. These

Plate I. Schizoid behaviour of an evaporating surface.
(Hickman, K. C. D., (1952) *Ind. Eng. Chem.*, **44**, 1892.)

authors have suggested that under quiet surface conditions the surface layer is depleted of the lowest boiling component of the charge at a faster rate than it can be replenished by diffusion from the body of the liquid.

Hickman and Trevoy (1952) have found that the apparent relative volatility of the binary mixture ethyl hexyl phthalate—ethyl hexyl sebacate distilled at high vacuum from a pot-still under a wide range of conditions, was greatly increased by agitation of the charge by mechanical stirring or an air inlet. The effect was so striking that they concluded that there is little reason not to use

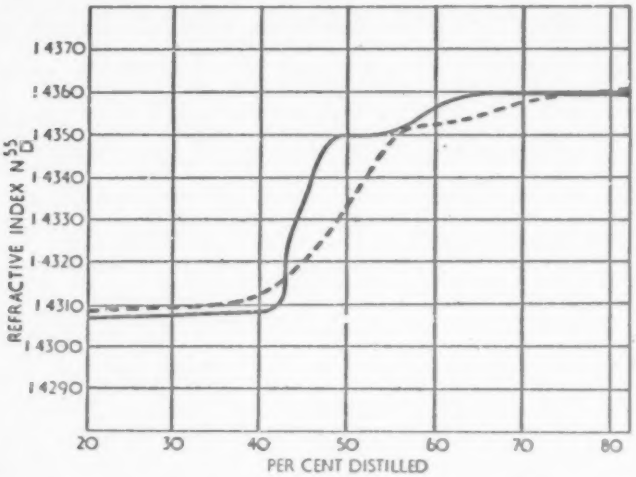

Fig. 1. Fractionation of methyl esters of hydrogenated rape seed oil fatty acids with (—) and without (. . .) stirring of the charge.
(from Birch, Gripp, and Nathan, 1947.)

agitation during distillation. Furthermore they have observed large differences in vapour composition from "torpid" and "working" surfaces. Thus for the above binary mixture at 150°C the apparent relative volatility from the working surface was 2·68, but only 1·25 from the torpid area. Since, as they have noted, the formation of a torpid surface is brought about by conditions causing depletion of the evaporating surface of its lighter components, and is further favoured by even heating of the still pot, it seems evident that the effect of stirring on fractionation noted by Birch, Gripp, and Nathan can be explained by the occurrence of "torpidity."

It is concluded from this recent work that stirring of the charge is essential for the greatest efficiency in low pressure fractionation.

3. *The efficiency of fractionation at low pressures*

Byron, Bowman, and Coull (1951) have considered in a qualitative manner the effects of pressure reduction on the efficiency of contact rectification. Besides difficulties associated with a large pressure drop, the vaporization of the charge, and high vapour velocities which may seriously interfere with

operation, they recognize several effects which concern the enrichment mechanism itself. These are caused by the increase of diffusion in the vapour phase and by the decrease of absolute vaporization and condensation rates at the interface with reduction of pressure. They are, firstly, the diffusion rate normal to the interface is increased and this increases the transfer rate between liquid and vapour and is favourable. Secondly, the diffusion rate in the direction of vapour flow is also increased and this effect may be strongly unfavourable. Thirdly, the decrease of the vaporization and condensation rates is unfavourable because it directly affects the transfer rate between phases. In considering the sum of these effects these authors have concluded that contact rectification cannot be expected to yield sharp separations at high vacuum, when recourse must be made to "multiple redistillation" for efficient fractionation.

Bowman and Tipson (1951), who also have described the above effects, state that no critical lower pressure limit for the effective use of contact rectification can be given, since the relative effects depend greatly on the design of the apparatus, but from practical experience atmospheric-pressure stills will function satisfactorily down to a pressure of a few centimetres. Also, in the range between about 1 mm and molecular-distillation conditions contact rectification cannot be employed to any useful extent. It seems obvious that these conclusions applied only to packed columns, but even so the pressure limit of a few centimetres is too high. It is possible to use packed columns of high efficiency down to head pressures of a few millimetres, at reduced boil-up rates and many examples are contained in the literature on lipids of such a practice. Weitkamp (1945) used a modified Stedman column, a typical atmospheric pressure type, for the effective separation of the wool wax acid methyl esters at a head pressure of 1·95 mm.

There appears to be no published work on the testing of column packings by binary mixtures at pressures below approximately 20 mm where the H.E.T.P. has been observed to be not widely different from that at atmospheric pressure (Hawkins and Brent, 1951; Berg and Popovac, 1949; Myles *et al.*, 1951). However, a number of determinations of the efficiency at lower pressures have been made on columns of small pressure drop, chiefly of the spinning band type. Zuiderweg (1952) found that a spinning band column lost 70 per cent of its efficiency between 760 mm and 10 mm, but Björkman and Olavi (1946) observed practically no decrease in the number of theoretical plates for a spinning band column of similar size, on reducing the pressure from 760 to 9 mm.

Several investigators (Williamson, 1951; Cruthirds, Jones, and Seyfried, 1951; Cowley, 1953) have determined efficiency also of spinning band columns at atmospheric pressure and head pressures of 1 mm and 0·05–0·1 mm, and Williams (1947) has compared the performance of an open tube wetted wall column at atmospheric pressure and 1 mm. Even though the accurate determination of H.E.T.P. at low pressures is attended with difficulties (see later), the combined results of the above workers are sufficiently pronounced and consistent to show a decrease of fractionating efficiency from that

at atmospheric pressure to $\frac{1}{2}$ — $\frac{1}{3}$ at 1 mm and $\frac{1}{2}$ — $\frac{1}{4}$ at 0·05–0·1 mm head pressure.

This fall in efficiency at low pressures, which is expected for the reasons given earlier, by no means renders contact rectification useless below 1 mm as suggested by Bowman and Tipson (1951). Reasonable efficiency at a head pressure of 0·5 mm is shown by the excellent separations made by Birch, Gripp, and Nathan (1947) and Murray (1951) with spinning band columns operating over their entire length below 1 mm. Under certain circumstances, such as for the fractionation of highly unsaturated esters, worthwhile fractionation might be achieved in wide spinning band columns of very small pressure drop down to a pressure of 0·05 mm, where Cowley (1953) has found the efficiency of such a column (36 mm diam.) to be approximately $\frac{1}{4}$ of its value at atmospheric pressure.

Until further necessary data on the efficiency of low pressure distillation is forthcoming it is concluded that there is loss of column efficiency at low pressures, but that for practical purposes the lower limit of useful operation for a column will probably be governed by its pressure drop.

B. Equipment

1. Columns and packings

Until recently practically all low pressure fractional distillations in lipid chemistry were performed with packed columns, which were probably selected on the basis of their high efficiency at atmospheric pressure. In general, packed columns containing a large number of plates operate with a large pressure drop, and the limitation which this places on their use at low pressures has already been stressed. However, it is obvious from their widespread use that packed columns fulfil many of the requirements of lipid chemists, especially for work of a routine nature, when a column of moderate efficiency would be adequate for the purpose.

Many types of packed columns have been used at low pressures but a general lack of comparable pressure drop data prevents an accurate assessment of their relative merits on this basis. However, Fenske glass helices (Longenecker, 1937) and the Stedman gauze cone packing (Weitkamp and Brunstruum, 1941), both widely used for fatty acid fractionation, are stated to have relatively smaller pressure drop characteristics. Fenske helices, suitable for columns of moderate efficiency, are readily available and relatively inexpensive. A modified Stedman packing more easily constructed than the original type has been described by Weitkamp and Oblad (1943) and used extensively by Weitkamp and co-workers (see later). Mitchell and O'Gorman (1948) have described a simply made but efficient helical-helix type of packing, a modification of which described by Shorland (1952) has a moderately small pressure drop.

To fill the need for a column of lower pressure drop than possible with any type of packing, Birch, Gripp, and Nathan (1947) have developed the spinning band column of Lesesne and Locke (1938). Their column of 36 mm diameter

had an operating pressure drop at 0·5 mm head pressure of only 0·04 mm or 0·002 mm per theoretical plate. With this column and excellent auxiliary equipment they demonstrated the possibility of precise fractionation of fatty acid esters at 0·5 mm or less. WILLIAMSON (1951) has since described a spinning band column of suitable size and efficiency for routine fractionation at 1 mm. A column of the same type described by MURRAY (1951) has been used for the fractionation of long chain wax components hitherto impossible to distil without at least serious risk of decomposition (MURRAY and SCHOENFELD, 1951, 1952, 1953) as well as for routine ester fractionation of fatty acids (HATT and SZUMER, 1953).

The spinning band type of column has very little hold-up which makes it most suitable for analytical fraction, and for fatty acid ester distillation this was demonstrated in a striking manner by WYMAN and BARKENBUS (1940). They effectively separated known mixtures of fatty acid esters using charges of only 1 to 5 gm. Because of their importance as general analytical columns, many designs have been published which mainly incorporate refinements on the original design of LESESNE and LOCHTE (1938). Recent well designed assemblies suitable for use at low pressures are those of CRUTHIRDS, JONES, and SEYFRIED (1950) and two commercially available models, the Podbielniak "Heli-Band,"* and the "Piros-Glover" micro still.† The difficulty in providing a trouble-free vacuum seal for the rotor shaft has been overcome by the use of a magnetic drive (FOSTER and GREEN, 1952).

The performance of the rotating concentric cylinder column (WILLINGHAM *et al.*, 1947; JOST, 1948) is outstanding at atmospheric pressure, combining a high boil-up rate without loss of efficiency with small hold-up and pressure drop. This last feature has suggested its use at low pressures, and its performance will be matter of some interest especially with the modified rotors equipped with reflux spreading attachments recently described by JOST, SIEG, and BRANDT (1953).

A new type of column, operating on the principle of "thermal rectification" has been described by BYRON, BOWMAN, and COULL (1951) and HICKMAN (1952). It consists essentially of a heated tube, containing a cooled, concentrically placed rotor. Fractionation takes place between the two by partial evaporation from the reflux film flowing down the heated walls, the condensed material being flung back by the rotor. Unevaporated residues flow downwards to the still pot, while vapour continually passes upwards to regions of lower pressure. This column has been developed into a small commercial unit by BENNER, DINARDO, and TOBIN (1951). A necessary feature has been added by PERRY and MANSING (1951) who maintain even distribution and stirring of the reflux film by a brush attached to the rotor. On account of its small pressure drop this column has been suggested for use at pressures down to a fraction of a millimetre. However, the writer has confirmed the observation of BENNER, DINARDO, and TOBIN (1951) that it is very difficult to operate, and for work

* Obtainable from Podbielniak Inc., Chicago, Illinois, U.S.A.
† Obtainable from H. S. Martin & Co., Evanston, Illinois, U.S.A.

on wax constituents has preferred a spinning band column of comparable pressure drop. The fractionating molecular still of TAYLOR (1951) may be considered a modification of this type in which the pressure drop has been eliminated. Upward movement of vapour is avoided by directing condensate to a higher point in the reflux film with the aid of upward slanting attachments on the rotor.

2. Accessory equipment

Efficient fractional distillation at low pressure depends not only on a suitable column but also on good accessory equipment. These include efficient insulation for the column, a still head incorporating either a constant take-off device or a reflux divider, means for control and measurement of head pressures and for measurement of head and still-pot temperatures, a pressure drop manometer between still pot and still head, and as stressed previously, a stirring mechanism for the charge.

It is customary to select or modify such equipment, which is largely interchangeable among column types, from the numerous designs in the literature, according to the particular requirements or resources of the investigator. The following remarks based mainly on the author's experience are intended partly to supplement recent reviews, as well as stress some points found to be of importance in the distillation of fatty materials.

(*a*) *Column insulation*—Because of their low boil-up rate and the occurrence of reflux as a thin film, spinning band columns (and other wetted wall columns) need better column insulation than packed columns. This is particularly so for columns of smaller diameter in which the provision of an efficient vacuum jacket is almost essential.

It is generally agreed that a vacuum jacket when well constructed is the most effective way of insulating a column, but their fabrication is beyond the resources of most laboratory workshops. One of the main difficulties is encountered, especially with large jackets, with the "baking out" process under vacuum and subsequent sealing off. For permanent installations this can be obviated by connecting the jacket permanently to a small diffusion pump for continuous evacuation during use (DOSTROVSKY and JACOBS, 1947). Perhaps the most popular alternative to vacuum jackets has been the use of electrical heater windings between two layers of lagging (asbestos, magnesia-asbestos or glass fibre). Adiabatic conditions are maintained by adjustment of the temperature in the vicinity of the windings to that of the column. Control is exercised through suitably placed thermocouples or differential thermocouples; it may be made automatic by the arrangement of HIGHET *et al.* (1950). The use of air thermometers in place of thermocouples for control purposes is suggested by DIXON (1950).

(*b*) *Still head and reflux ratio control*—GIVEN (1950) has stressed that the multitude of designs of still heads of the total condensation type are necessarily of two types, either those which, independently of boil-up rate provide for a constant reflux ratio, or those which provide for a constant rate of take-off. None

can do both although this is achieved when the boil-up rate is kept constant. For low pressure distillation designs incorporating constant take-off are usually preferred. They allow reflux ratio to be varied over a wide range during operation, fractions of equal size may be collected at regular intervals, and their relatively simple designs allow for easy lagging or heating to prevent solidification of distillate. However, the use of glass stopcocks for the regulation of the take-off of distillate should be avoided. The need to heat them renders them a source of leakage of air into the system and also of contamination by the stopcock lubricant. The better designs employ glass or metal needle valves for this purpose. For very low take-off rates such as is necessary with spinning band columns, very fine adjustment may be avoided by opening the valve at short intervals by operation through a solenoid and timing device. To maintain a constant reflux ratio constant boil-up is best achieved by adjustment of the still-pot heater to give a constant pressure drop.

(*c*) *Measurement of head temperatures*—A true record of head pressure and head temperature is of undoubted value (see below), but it has often been noted that temperature readings are inconsistent at low pressures and especially below 1 mm and at low boil-up rates (Hilditch, 1947; Birch, Gripp, and Nathan, 1947; Williamson, 1951). This is probably due to the poor heat transfer between vapour and the thermometer or thermocouple, which allows radiant heat effects to or from the column walls or heating jackets to become appreciable. Birch, Gripp, and Nathan (1947) eliminated these effects by surrounding the thermometer bulb with a polished metal shield and by lagging of adjacent column walls. Murray (1951) has guarded against radiation from heated column jackets by wrapping the thermocouple well with aluminium foil.

Head temperature records of pure compounds are only of value if the pressure recorded is indeed that at the still head. Wide bore connections of short length between column head and manometer should be used so that flow of vapour does not create an appreciable pressure drop. For this reason mechanical stirring of the charge is preferred to the use of a gas leak. Condensible vapours arising from decomposition should be removed by a dry ice or liquid air trap close to the head. It is highly likely that the use of narrow connections between still head and manometer combined with the use of a gas leak for stirring of the charge is the main reason for the wide variation in recorded boiling points at low pressures.

More precise recording of head temperatures at low pressures (to $\pm$ 0·2°C) with the use of fine pressure control (see below), and observance of the above factors now makes boiling point at low fixed pressures of added significance for the identification of organic compounds. This is specially so for homologous series of fatty compounds where the difference in boiling point is more pronounced than with the melting point. For the easy comparison of data, boiling points should be recorded only at certain standard pressures (e.g. 0·5, 1, 2, 5, 10 mm).

(*d*) *Pressure control*—Of the many manostats which have been described, only a few are reported to operate with precision and reliability at pressures of 1 mm

or less (WILLIAMS, 1947; BIRCH, GRIPP, and NATHAN, 1947; ROMANET, 1950). Of these the simplest and probably the most reliable is that described by BIRCH, GRIPP, and NATHAN, 1947 (see WEISSBERGER, 1950, p. 285). Gases from the column pass through an adjustable head of oil which governs the column pressure, and so to the pump which provides a reference pressure. The pump performance must therefore be consistent and along with the manostat should be protected by a cold trap against traces of solvents and decomposition products. Under such conditions control to within $\pm$ 0·01 mm is possible and consistent over long periods without resetting. A manostat similar in principle has been described by TODD (1948) and is available commercially.* Filled with butyl phthalate it has a pressure range up to 4 mm and also a sensitivity of $\pm$ 0·01 mm.

(*e*) *Fraction cutters*—BIRCH, GRIPP, and NATHAN (1947) have described a fraction cutting device which eliminates most of the defects of earlier conventional designs. It is compact and therefore simple to warm, it contains no grease lubricated stopcocks, and it permits an indefinite number of fractions to be taken. If it is used with an additional pump no air need be admitted to cause fluctuation of column pressure.

(*f*) *Stirring and heating of the charge*—From the point of view of decomposition the last stages of a distillation are naturally the most hazardous. In an externally heated round bottom still pot, under conditions of quiet evaporation the surface area of the charge decreases as the charge evaporates. The smaller boil-up rate due to this decrease must be compensated by an increase of the temperature of the heating bath. The additional heat dries out of the wall of the still pot, raises it above the temperature of the liquid and can thereby cause considerable decomposition, especially at the meniscus. Such conditions which appear quite likely with much of the distillation equipment in current use can be avoided by stirring the charge, preferably with a stirrer which can maintain a large area of fresh surface until the charge is almost completely evaporated. Such a stirring device may easily take many forms but the simple conical and tubular centrifugal stirrers used by HICKMAN and his associates in their high vacuum still designs are most suited to this purpose (e.g. see HICKMAN and TREVOY, 1952; TREVOY and TORPEY, 1953). A stirrer of this type made entirely of stainless steel and adapted for use with a spinning band column is shown in Fig. 2. The charge is pumped by the inclined tubes *A* from the lowest level of the flask and thrown outwards to the walls of the flask there to flow down as a film. The tubes *A* are mounted on a strip of stainless steel *B* (0·005 in. thick) which allows the device to collapse for removal with the band through the column. The amount of charge remaining unevaporated after pumping ceases depends only on the clearance of the inclined tubes with the bottom of the flask and can be made quite small. For use with packed columns such a device could easily be operated through the simple vacuum shaft seal described by TREVOY and TORPEY (1952) or by a magnetic drive.

For rotor columns the possibility also exists of making the lower part of the

* Todd Scientific Company, Springfield, Pa., U.S.A.

column into a film evaporator by an arrangement shown in Fig. 3. The charge is pumped from the extreme bottom of the pot through a well-fitting square-threaded screw revolving at rotor speed in a stationary tube. It is flung to the column walls where it joins the reflux and flows down filmwise over the evaporating section. This arrangement has the advantages (*a*) that only a

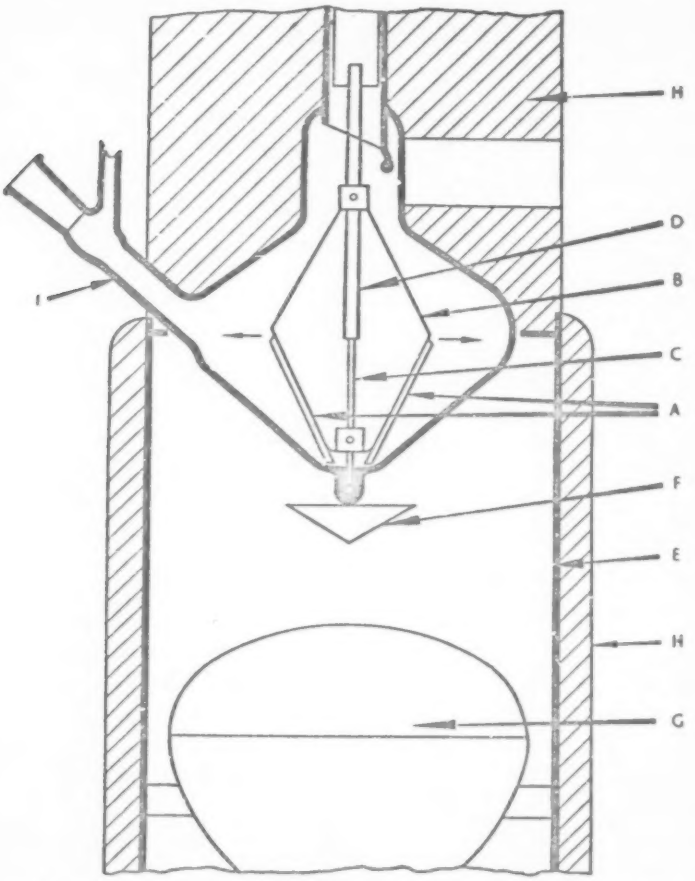

Fig. 2. Centrifugal type of stirrer in a spinning band column, *A*—inclined tubes, *B*—flexible support for tubes *C*—solid spindle loose fit in hollow spindle (*D*), *E*—aluminium walls polished internally, *F*—conical shield to deflect direct radiation, *G*—infra red lamp, *H*—lagging, *I*—side arm (thermocouple well omitted).

fraction of the charge is in the evaporating zone at any time: the pot may be considered a reservoir and kept at a safe lower temperature, (*b*) heating of the evaporating section at all stages may be mild as a large and constant area of flowing surface is maintained until the pumping ceases. This evaporator has worked well when used with the rotary condenser column of Benner, Dinardo, and Tobin (1951) (the author, unpublished data).

The heating of still pots is usually by means of electrically heated air or oil baths or by glass cloth electric heating mantles. With such external heating, heat must be conducted through the walls of the still pot and a considerable heat gradient may be set up between the external heating medium and the evaporating surface. Although this gradient may be largely reduced in the

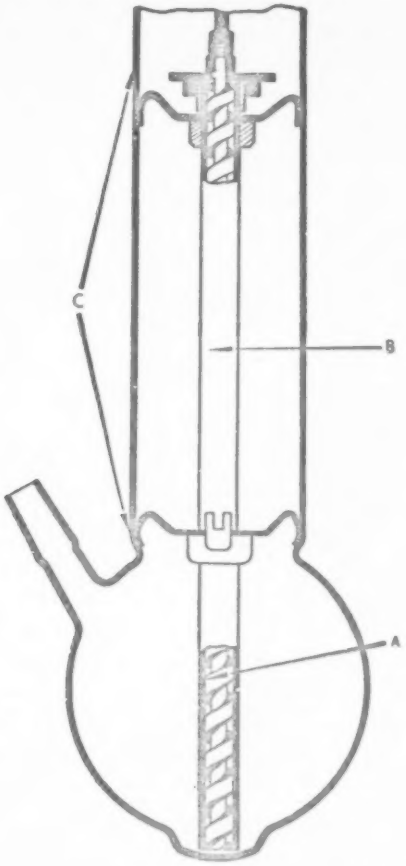

Fig. 3. Film evaporator. *A*—rotating screw, *B*—stationary tube, *C*—electrically wound section.

charge by efficient stirring, the walls of the pot must of necessity be at a higher temperature and decomposition when it sets in could be expected to be initiated in their immediate vicinity.

Reavell (1944) first suggested that the heat barrier created by the poorly conducting (still pot) walls could be eliminated by the use of infra-red radiation for heating, making the containing walls of materials transparent to this radiation. Heat is thereby absorbed directly by the charge and high wall temperatures are avoided. With the ready availability of industrial infra-red

heating lamps in recent years, a number of investigators have used them for heating laboratory distillation equipment (Brasch, 1948; Lautie and Moutet, 1949; Booy and Waterman, 1949). Rose (personal communication) has applied this method of heating to the fractionation on a large laboratory scale of fatty acid methyl esters. He used a number of lamps and the glass pot was equipped with a novel "bouncing" stirrer which promoted even evaporation by violent disruption of the surface of the charge. With such modifications decomposition was reduced in a most impressive manner. This beneficial effect of infra-red heating on decomposition has been verified (the author, unpublished data) when fractionating compounds of longer chain length in a spinning band column equipped with the heating-stirring arrangement shown in Fig. 2. Furthermore, superheating of the pot contents was observed to be virtually eliminated, as the difference in head and pot temperatures of the above column, when distilling a pure compound, exactly correspond to the difference in pressures as measured by the pressure drop. At 2 mm head pressure a difference of only 4°C was recorded.

It is concluded that infra-red radiant heating has considerable advantages over the orthodox methods. Apart from the above reasons, it is safe, has almost immediate response to changes in input, permits a clear view of the charge, and the lamps are easy to install or replace.

C. Column operation

1. Column testing at low pressures

In recent years a number of binary mixtures have been investigated for the determination of H.E.T.P. at pressures in the region of 1 mm and less (Williams, 1947; Perry and Fuguitt, 1947; Benner, Dinardo, and Tobin, 1951; Trevoy, 1952). These mixtures may give valuable data especially for comparison between column types, and for variation of operating conditions, but from the point of view of actual column operation, results from their use should be accepted with some reservation because of the following reasons: (*a*) The binary mixture may have quite different film-forming properties to the materials to be regularly distilled. (*b*) It is necessary to assume that the relative volatility α is constant over the whole pressure and temperature range of the column. (*c*) Doubt may be cast on the accuracy of earlier data by the discovery of Hickman and Trevoy (1952) that α may vary widely according to the evaporative conditions prevailing in the equilibrium still. Furthermore, H.E.T.P. determinations are usually made under equilibrium conditions at total reflux, a condition never attained under working conditions. The most reliable method of judging the efficiency of a column for any particular use is from the sharpness of separation of model compounds revealed by a distillation curve (product composition — per cent distilled) determined under operating conditions (*cf.* Rose and Rose, 1951). The model compounds should be closely related to those to be regularly distilled, as differences in film-forming properties may exist. It is noted, for example, that long chain fatty acids (Benner,

DINARDO, and TOBIN, 1951) and long chain alcohols (MURRAY, 1951) do not wet hot column walls well enough to give a reflux film.

2. Operating pressure

The probable loss of some of the column efficiency at low pressures already considered, naturally makes it desirable to operate at as high a head pressure as possible without decomposing the charge. However often the possibility of decomposition makes it desirable to operate at the minimum pressure possible. As a general rule this should not be much less than a value equal to the pressure drop of the column. Further decrease of head pressure should be made only when the measurement of pot temperatures indicates that advantage is to be gained.

3. Reflux ratio

It is the usual practice for the most efficient operation of a column to use a reflux ratio approximately equal to the number of plates in the column, and ROSE (see ROSE and ROSE, 1951, p. 169) has shown that this has a theoretical basis. He has concluded that to achieve a relatively good separation (his "standard separation") with the most effective use of the plates in a column (and of the time consumed), the reflux ratio should be not less than $\frac{2}{3}$ or more than $\frac{3}{2}$ of the number of plates. Within these limits a lower number of plates may be compensated by a higher reflux ratio and vice versa, but little benefit may derive, in terms of sharpness of separation, from operation outside these limits. Thus a column of 20 plates gives substantially the same separation when the reflux ratios is increased from 40 to 80 : 1.

Since the time of distillation is of prime importance in low pressure distillation because of decomposition, and this is controlled by the reflux ratio, it is naturally desirable to operate at as low a reflux ratio as possible without affecting the sharpness of separation. It is therefore preferable, if its pressure drop is not excessive, to use a column of greater efficiency than necessary so that the desired separation is achieved with a reflux ratio as low as possible. This is so when, as is quite common, one column of high efficiency is used for all low pressure fractionations in the laboratory. The reflux ratio may then be adjusted according to the difficulty of the particular separation. With the relatively easy separation of homologous fatty acid esters quite low reflux ratios are then permissible. Thus WEITKAMP and BRUNSTRUUM (1941) obtained excellent separations with a reflux ratio as low as 3 or 4 : 1 provided this was raised as the transition points were approached. However, when no clear indication of the early approach of a transition point can be obtained, as when the column is insulated with a vacuum jacket, the use of a constant adequate reflux is advisable.

4. Boil-up rate: charge: chasers

The boil-up rate (or throughput) directly affects the time required for a distillation, and should therefore be as high as possible. However, its increase

raises the pressure drop, usually causes a loss in efficiency, and eventually for packed columns, the onset of flooding. The rate chosen can therefore only be a compromise between the time available and the demands of these factors. For packed columns the boil-up rate at low pressures must be considerably less than the optimum value at atmospheric pressure. Spinning band columns must be operated at low boil-up rates as their high efficiency rapidly decreases with increase of boil-up rate.

There seems to be little theoretical guidance as to the optimum size of the charge for any particular column, but for practical purposes it is related to the operating hold-up and usually a volume of 10 to 20 times this value is recommended. This means that when the distillation is finished, 5 to 10 per cent of the charge remains undistilled. These "column residues" may be readily obtained as distillate by the addition to the charge of a higher boiling material which "chases" the last of the charge through the column. The chaser should be stable and must be easily separable from the material under examination as it will be present with it in the last fraction. The possibility of successfully distilling small charges with the aid of a chaser has been demonstrated by PODBIELNIAK (1945). He obtained remarkable fractionation with a charge approximately equal to the column hold-up, and a column having more theoretical plates than was necessary for the separation. This use of a chaser should be borne in mind, particularly for routine fatty acid ester fractionation, where often only a limited quantity of esters is available.

Probably the best selection of a chaser for fatty acid ester fractionation would be from a series of hydrocarbons of suitably spaced boiling points. As such compounds are not readily available a fraction of a commercial mineral oil may be substituted. The oil may be conveniently fractionated into 10° cuts and that fraction used which boils 20 to 30° above the boiling point of the highest ester likely to be present. The end of the ester fractionation is then marked by the sudden rise in head temperature to the boiling range of the chaser. Hydrocarbon occurring in the last ester fractions may be easily separated either by crystallization, or by selective adsorption on neutral alumina of silica, or the free acid may be isolated after hydrolysis.

III. Fractional Distillation of Fatty Acid Methyl Esters

1. Simple fractional distillation

The analytical fractionation of fatty acid methyl esters by simple fractional distillation (as distinct from amplified distillation) is perhaps the most common operation in the analysis of fats and oils, but is frequently performed in such a way as to result in very poor fractionation. The object, to divide the esters into families differing in the degree of unsaturation but of the same chain length, is thereby not accomplished and individual fractions may contain esters of 2 or even 3 chain lengths. It is true that satisfactory analyses can be made on such fractions, on the basis of their chemical constants and ultraviolet absorption measurements, but the resulting calculations are involved, and

require several simplifying assumptions. Great (and perhaps undue) reliance must also be placed on the accuracy of the above determinations.

Good separation between esters of even carbon number using columns of moderate to high efficiency is a relatively simple matter as many workers have shown. A large proportion of the fractions (the plateau fractions) are obtained of a single chain length, which makes for simplified and more accurate subsequent analysis. The number of fractions taken need be few, one for each plateau and one for each transition. Good fractionation decreases the possibility of overlooking the presence of minor constituent acids, and permits the positive identification of the major ones by their isolation from the plateau

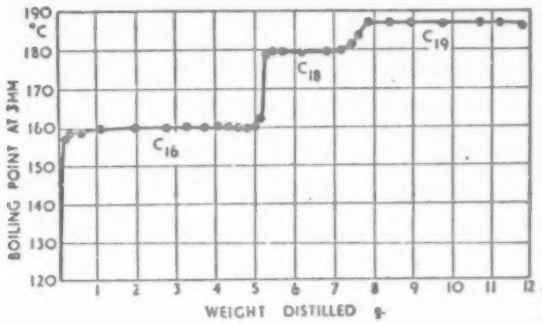

Fig. 4. Semi-micro fractionation of the methyl esters of the acids from *Lactobacillus arabinosus*.
(from HOFMANN, LUCAS, and SAX, 1952.)

fractions in a state of purity. WEITKAMP and BRUNSTRUUM (1941) have considered that precise fractionation is in itself sufficient for a reasonably accurate analysis of fully hydrogenated homologous esters, the composition being determined from the mid points of the temperature transitions between plateaux.

A fine example of the efficient use of simple distillation and modern equipment is supplied by recent work of HOFMAN, LUCAS, and SAX (1952). From the lipids of Lactobacillus arabinosus they have isolated on a semi-micro scale a new and unusual C_{19} acid containing a cyclopropane ring. A semi-micro spinning band column (Piros-Glover) was employed at 3 mm head pressure. The distillation diagram is shown in Fig. 4 and the excellent separation of the C_{19} methyl ester from the C_{18} esters is noteworthy. With the aid of precise fractional distillation techniques, SHORLAND and his co-workers have detected and isolated small percentages of hitherto unsuspected branched chain fatty acids from a number of common animal fats (HANSEN, SHORLAND, and COOKE, 1951, 1952a, 1952b, 1953; HANSEN and SHORLAND, 1951, 1952; MORICE and SHORLAND, 1952). The diversity of the fats (ox, mutton, butter, shark liver oil) suggests that distribution of such branched acids is widespread in fats and because of their small amounts have been hitherto overlooked. CASON and SUMRELL (1951) have reported that by repeated simple distillation of a sample of methyl phthioate, that phthioic acid previously considered a chemical entity,

is indeed a complex mixture of at least twelve components, one of which (C_{27} phthienoic acid) was isolated in a pure state.

2. Amplified distillation

WEITKAMP (1947) has pointed out certain limitations of fractional distillation for analytical work, and to overcome these he has described a technique of amplified distillation with special application to the fractionation of fatty acid methyl esters. Quoting from his paper:

> "If a large number of theoretical plates is required for a given separation, it follows that a correspondingly large amount of a given component must be present before any is available for separation at the desired purity. The reason for this is that as a cut point is approached and passed, there is a gradual change in the composition of the distillate from lower boiling component to higher boiling component. The amount of this inseparable mixture is a function of the column hold-up per theoretical plate.
>
> "Amplified distillation is a technique by which the interjacent mixtures inevitably encountered in ordinary fractional distillation may be eliminated in certain specific instances. This permits a more nearly complete separation of the components in the desired purity and is especially useful in case certain components occur in small proportions or in case the sample is of limited availability. Amplified distillation does not alter the efficiency of a given still but merely allows the full efficiency of the still to be developed with respect to the separation of each component, regardless of the amount or proportion present in a given mixture.
>
> "In amplified distillation an additional component is introduced. The component is so selected that it distills between the components of the mixture and is easily distinguishable and separable from them. For the amplified distillation of methyl esters a hydrocarbon mixture of petroleum origin meets these requirements and has the added advantages of a smooth true boiling point curve and ready availability. It is only necessary to select a commercial product having the appropriate distillation range."

WEITKAMP has found that the distillation of free fatty acid mixtures with mineral oil gives indifferent separation of only qualitative value. Due to azeotrope formation they are completely eliminated below their boiling points. Azeotrope formation is not encountered when using the relatively non-polar methyl esters, which distil at their normal boiling points. Their elimination curves closely follow the shape of Maxwell distribution curves which are shown superimposed on them in Fig. 5. The peak of the curve corresponds with the true boiling point of the methyl ester. Like the distribution curves, the elimination curves extend considerably in both directions but overlapping may be so slight, as between the C_{16} and C_{18} curves in Fig. 5, that acids of better than 99 per cent purity may be isolated from these fractions.

A striking example of the degree of separation which can be achieved by this method of distillation of fatty acid esters is provided by its application by

WEITKAMP (1945) to the separation of the acids of wool wax. No less than 32 acids were isolated in a state of considerable purity. They belonged to four homologous series (normal-, iso-, ante-iso, and 2-hydroxy-) and the separations are all the more remarkable in that the corresponding normal and iso acid esters boiled only 3 to 4° apart, and many of the acids were present only in traces. This work undoubtedly deserves the tribute of being the finest example of the effective use of distillation methods in the history of investigative work on lipids. Amplified distillation has also been employed by WEITKAMP and co-workers for the isolation of the acids of human hair fat (WEITKAMP, SMILJANIC,

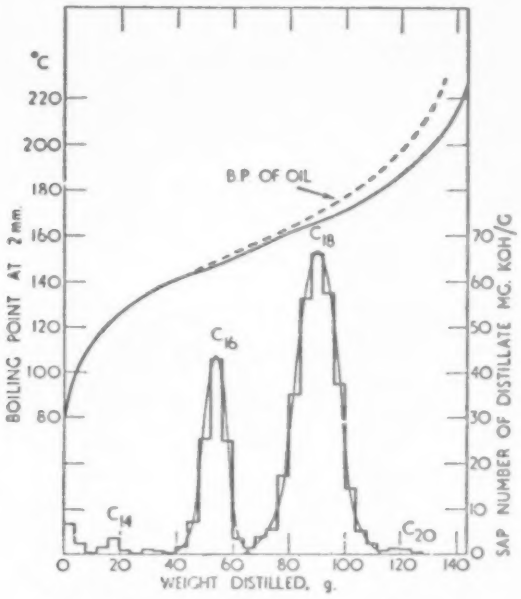

Fig. 5. Amplified distillation of cottonseed oil methyl esters (from WEITKAMP, 1947.)

and ROTHMAN, 1947; ROTHMAN *et al.*, 1947). It is indeed surprising that it had not been used for the more usual analysis of fatty acids from fats and oils until only recently, when HATT and SZUMER (1953) employed it in conjunction with precise simple fatty acid ester fractionation. They considered that the information available from the amplified distillation of the hydrogenated and saturated esters was, where applicable, more reliable than that provided by simple fractionation.

BARRY, SATO, and CRAIG (1951) have pointed out that a satisfactory method for use in biochemical work for the separation of homologous fatty acids should meet the following requirements: (*a*) it should give good resolution between the members of the series, (*b*) regardless of their relative amounts, (*c*) be quantitative, and (*d*) provide sufficient material for identification by the classical

methods. These authors further considered that precise fractional distillation satisfactorily fulfilled these conditions except that it usually required too much material and for this reason attention of investigators was being diverted to the possibilities of other separation processes based on chromatography and counter-current solvent distribution. This objection on the score of material needed is largely removed by the use of the amplified distillation technique which furthermore fulfils the other requirements above even better than simple fractional distillation. WEITKAMP (1947) has stated that excellent analyses can be performed on a fraction of a gramme of methyl ester mixture. With the

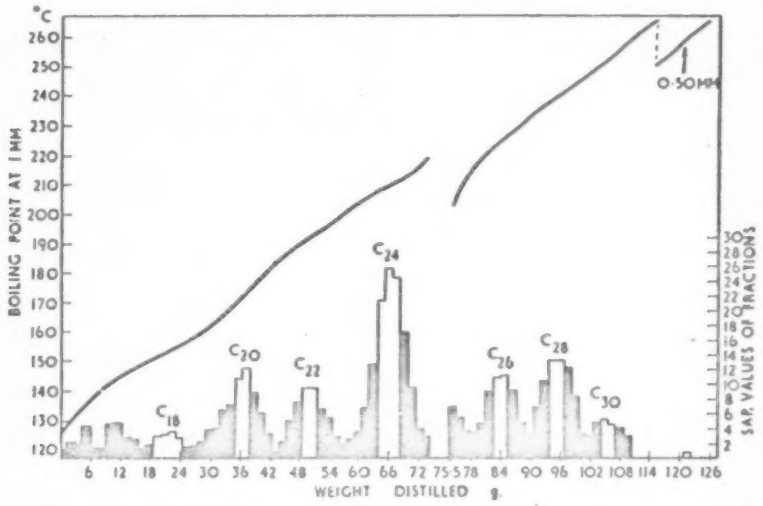

Fig. 6. Two-stage amplified distillation of the methyl esters of the normal acids of carnauba wax.
(from MURRAY and SCHOENFELD, 1953.)

recent development of efficient micro columns the ester charge could be made very small, particularly when in special cases (such as for the normal saturated acids), identification could be made on the boiling points of the esters, as shown by the peaks of the elimination curves. The limitation for accurate quantitative analysis in this case would probably be the accuracy of the micro saponification determination on the fractions. The value of the amplified distillation to biochemical work is well illustrated by its effective use in the separation and total recovery of small amounts of labelled carbon fatty acids derived from experimental animal tissues (MASORO, CHAIKOFF, and DAUBEN, 1949; DAUBEN, HOERGER, and PETERSEN, 1953).

Amplified distillation, combined with the use of an efficient spinning band column, has been shown to be a potent combination for the separation of long chain wax constituents. MURRAY and SCHOENFELD have found that acetylated long chain alcohols can be separated in a similar fashion to the acid methyl esters, and with the above combination have isolated the normal alcohols of

carnauba wax (1951) and branched (iso- and ante-iso-) alcohols from wool wax (1952). Further work (MURRAY and SCHOENFELD, unpublished data) has shown that homologous series of long chain diols and hydroxy acids may be likewise successfully separated as their diacetates or acetylated methyl esters respectively. They have also separated seven normal acids from carnauba wax (1953) a record of the amplified distillation being shown in Fig. 6. It is noteworthy that from an ester charge of only 5 gm, seven acids could be isolated (from the unshaded fractions) in a state of purity sufficiently high to make their positive identification by crystal form, x-ray spacings and melting points, a relatively simple matter.

CASON and SUMRELL (1951) have investigated the use of amplified distillation for the separation of fatty acids of bacterial origin, and consider it of limited value because (*a*) the boiling point of the ester is lowered by the presence of the oil and (*b*) the efficiency of the column is likewise lowered. The claim (*a*) has been refuted by MURRAY and SCHOENFELD (1952) who confirmed WEITKAMP's original findings. Furthermore, the data quoted by WEITKAMP (1945) for the boiling points of methyl esters of normal acids isolated by amplified distillation, are in good agreement (within 1°C) of recent vapour pressure-temperature measurements on the pure esters (see later). Regarding their second claim (*b*), it is obvious from WEITKAMP's results that the efficiency of his column had been considerably improved with respect to the separation of adjacent esters (C_{16}, C_{18}): separation of such esters to the extent of 99 to 100 per cent would have been impossible by simple distillation with any column at present available. It is probable that the disappointing results of CASON and SUMRELL was a result of their use of a column of considerable lower efficiency than that of WEITKAMP.

3. *Fractional molecular distillation*

Molecular distillation as performed in the laboratory with a pot still or falling film apparatus has a relatively poor separating power. For this reason it is rarely used for the separation of fatty acid mixtures, although the work of FARMER and VAN DEN HEUVEL (1938) on the fish oil acids is a fine example of its effective use in this respect, at a time when other methods of distillation were not practicable.

In recent years a number of fractionating molecular stills have been described (WOLLNER, MATCHETT, and LEVINE, 1944; BREWER and MADORSKY, 1947; MADORSKY, BRADT, and STRAUS, 1948; BOOY, 1952). These stills consist of an inclined heated section and a colateral condenser and fractionation is by multiple redistillation between the two. Condensate from one stage flows by gravity to become the charge of the next higher stage and so on upwards, while unevaporated residues flow downwards. A vertical still (TAYLOR, 1951), already discussed, operates on the same principle, the movement of the condensate upwards being induced by the revolution of the condenser. While stills of the above types must necessarily involve a considerable time of contact of the material with the heated zone, they nevertheless offer the possibility of fractionating at considerably lower temperatures, fatty acid esters and other lipid

constituents which, because of their molecular weight or instability, cannot be fractionated at higher pressures.

4. Vapour pressure-temperature data

Vapour pressure-temperature measurements on purified methyl esters have been mainly confined to the esters of the straight chain acids of even carbon number. The earlier work has been summarized by MARKLEY (1947). Since then BONHORST, ALTHOUSE, and TRIEBOLD (1948) have measured the vapour pressures of the methyl, propyl, and isopropyl esters of the acids C_6 to C_{18} over a pressure range 2 to 100 mm or as much of this range as the stability of the esters permit. They used a method modified from that of NATELSON and ZUCKERMAN (1945). Only two drops of ester were required for a measurement. SCOTT, MACMILLAN, and MELVIN (1952) have reported data for the methyl esters of the acids C_8 to C_{18} over the range 0·1 to 6·0 mm obtained with a pot tensimeter similar to that of HICKMAN, HECKER, and EMBREE (1937). They did not make measurements above 200°C, accepting the statement of BONHORST, ALTHOUSE, and TRIEBOLD that decomposition occurred above this temperature. The purity of their esters was checked by careful determination of freezing point curves. STAGE (1953) has examined previous vapour pressure data of the whole series of fatty acid methyl esters C_1 to C_{18} over a wide pressure range. He has applied to this assembled data a graphical method of correction and estimation, in which the data for any one ester is considered in relation to that of the data of the whole homologous series (STAGE, 1951). The latter have been plotted in several ways and the relationships between the families of curves so obtained have been used to select or correct the existing data of any

Table 1. Boiling points of methyl esters of fatty acids

	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	
1·0 mm . . .	37·5	66·0	92·3	114·8	137·0	158·0	A
	37·9	66·0	92·0	114·5	137·3	158·2	B
2·0 mm . . .	48·0	77·0	103·7	127·0	148·9	171·4	A
	48·2	77·0	103·5	126·7	150·0	171·2	B
	51·5	78·7	102·8	127·1	148·5	169·4	C
4·0 mm . . .	59·5	89·3	115·9	140·0	162·9	185·3	A
	59·5	89·1	116·1	140·2	163·7	185·4	B
	62·5	90·8	116·0	141·3	163·2	184·6	C
6·0 mm . . .	66·6	96·2	121·6	148·8	172·0	194·3	A
	66·4	96·6	124·1	148·8	172·3	194·3	B
	68·9	98·1	124·4	150·3	172·6	193·6	C
10·0 mm . . .	76·0	106·6	134·0	160·8	183·8	206·0	A
	77·8	108·0	135·0	161·8	184·8	206·6	C

A. STAGE (1953).
B. SCOTT, MACMILLAN, and MELVIN (1952).
C. BONHORST, ALTHOUSE, and TRIEBOLD (1948).

particular member. New data has been obtained by extrapolation or interpolation. It is noticeable that his "ironed out" results for the higher esters closely follow the values of SCOTT, MACMILLAN, and MELVIN (1952). Table 1 reproduces the results of these investigations for methyl esters C_8 to C_{18} over a pressure range of 1 to 10 mm. Fragmentary vapour pressure data which are not included in this table have been published by NEVIN, ALTHOUSE, and TRIEBOLD (1951) and MONICK, ALLAN, and MARLIES (1946).

Accurate determinations of vapour pressure of methyl esters of acids of chain length greater than 18 carbons have never been published, although their boiling points under various low pressures have been observed by several investigators in the examination of natural sources of these acids. Table 2 is compiled with slight corrections for pressure from the data of WEITKAMP (1945) and MURRAY and SCHOENFELD (1953, and unpublished data).

Table 2. Boiling points of methyl esters of long-chain fatty acids

	C_{20}	C_{22}	C_{24}	C_{26}	C_{28}	C_{30}
1·0 mm . .	176	194	211	226	241	255
2·0 mm . .	189	206	223	238	253	

SCOTT, MACMILLAN, and MELVIN (1952) have obtained data for methyl oleate, linoleate and linolenate, prepared from the bromides. The results for the range 1 to 6 mm pressure are given in Table 3, and because of inconsistencies between the earlier data for methyl oleate and linoleate (ALTHOUSE and TRIEBOLD, 1944; NORRIS and TERRY, 1945) these are included for comparison. It is seen that the recent data for oleate and linoleate agree more closely with the data of ALTHOUSE and TRIEBOLD but that the oleate boils higher, not lower than the linoleate. This reversed order and the close boiling points now explains the

Table 3. Boiling points of unsaturated esters

	Methyl oleate	*Methyl linoleate*	*Methyl linolenate*
1·0 mm . .	154·4	154·0	155·0
	152·5[a]	149·5[a]	
2·0 mm . .	167·4	167·1	168·0
	166·5[a]	163·0[a]	
	166·2[b]	166·5[b]	
4·0 mm . .	181·7	181·3	182·2
	182·0[b]	182·4[b]	
6·0 mm . .	190·8	190·2	191·1
	192·0[b]	193·0[b]	

[a] NORRIS and TERRY (1945).
[b] ALTHOUSE and TRIEBOLD (1944).

observation of NORRIS and TERRY that only a slightly greater concentration of linoleate was obtained in earlier fractions when a linoleate-oleate mixture was fractionated in an efficient column.

With the isolation of many branched acids in recent years, the relation between branching and boiling point is of considerable interest. WEITKAMP (1945) found that the esters of the iso acids (C_{10} to C_{26}) boiled 3 to 4°C lower than the corresponding normal esters, at 1·95 mm pressure. CASON *et al.* (1951) have stated that in comparison with the boiling point of the normal isomer at 3·75 mm pressure, (*a*) a single methyl branch causes a lowering of approximately 5°C, (*b*) a n-hexyl branch 7°C, (*c*) three methyl branches have double the effect of one, and (*d*) a quaternary carbon (α or γ) causes a depression of as much as 22 to 23°C. These observations broadly follow the known effects of branching in the lower alcohols and hydrocarbons. Much precise information is given by the data of ROSSINI *et al.* (1947) for the vapour pressures of the paraffin hydrocarbons up to and including C_9. For methyl branching two effects are general, (*a*) a branch in the 3-position lowers the boiling point of n-nonane approximately 1° less than those in the 2- or 4-positions, (*b*) the effect of two or three methyl branches varies considerably according to their position.

5. *Thermal stability of fatty acid esters*

Although a knowledge of the thermal stability of various classes of fatty acid esters is of prime importance in fractional distillation, there is very little exact information on this point.

In the course of vapour pressure-temperature measurements on fatty acid methyl esters BONHORST, ALTHOUSE, and TRIEBOLD (1948) found no visible signs of decomposition even at temperatures above 250°C, but erratic vapour pressure readings above approximately 205°C led them to the conclusion that this was due to decomposition. It must be pointed out, however, that, owing to the presence of air in the apparatus, the nature and trend of these results might well be explained by oxidation of the ester sample, and indeed oxidation of the hydrocarbon chain might be expected at these temperatures in view of the relative ease with which higher hydrocarbons are oxidized by gaseous oxygen at even lower temperatures (see below). Furthermore, the pronounced drop in vapour pressure over a period of time (90 min) observed by these authors in the temperature range 208 to 240°C suggests extensive decomposition which is not borne out by the published details of many ester fractionations, in which pot temperatures have exceeded 250°C without extensive decomposition being reported. HATT (1948) has stated that methyl esters of saturated acids begin to break down rapidly at 300°C.

The decomposition temperatures observed by ALTHOUSE and TRIEBOLD (1944) for methyl oleate (217°C at 16 mm) and methyl linoleate (208°C at 11 mm) may also be subject to doubt as air was apparently not excluded from the apparatus. An indication that monoethenoid methyl esters may have a stability much greater than shown by this figure for methyl oleate may be gathered from the work of YOUNGS *et al.* (1951) who, in a precise fractionation

of the esters of rape-seed oil acids, recorded head temperatures as high at 250°C (methyl erucate) and reported only minor decomposition (0·5 per cent) which they attributed to diene and triene esters.

With increasing unsaturation thermally induced polymerization becomes of increasing importance which is demonstrated by many investigations on the highly unsaturated fish oil acids. Norris *et al.* (1943) studied the effect of heat on the methyl esters of the cod liver oil acids. Spectroscopic data showed that at 150°C conjugation and polymerization was very slow or negligible, while at 200°C it was slow but appreciable. Hilditch (1947) concluded from the results of Farmer and Van den Heuvel (1938) that fish oil methyl esters could not be heated above 200°C without change. The divergence of opinion regarding the extent of conjugation and polymerization during fractional distillation of the more unsaturated esters is due no doubt to the degree of severity of the conditions used. Thus it may be calculated from the rate of distillation used by Farmer and Van den Heuvel (1938), who abandoned orthodox fractional distillation for molecular distillation, that their distillation time of the esters of the acids of sardine oil extended to approximately 20 days! Norris *et al.* (1941), who obtained less conjugation of cod liver oil methyl esters, used a Longenecker column with a distillation time of only 8 hr. It needs emphasizing that with the development in recent years of efficient columns with small pressure drop, the idea that such esters cannot be successfully distilled is no longer valid. A spinning band column of the dimensions of that of Birch, Gripp, and Nathan (1947) can easily work efficiently within the safe temperature limits which the work of Norris *et al.* (1943) has shown to be desirable.

Oxidation has already been suggested as contributing to the breakdown of esters at moderate temperatures, and support for this is obtained from investigations of the oxidation of pure hydrocarbons by gaseous oxygen. From results obtained at 110°C Larsen, Thorpe, and Armfield (1942) concluded (*a*) that for saturated hydrocarbons the process is autocatalytic and (*b*) the presence of a double bond greatly reduces stability and seemed to be the first point of attack. Benton and Wirth (1953) autoxidized n-decane at 145°C under conditions that 80 per cent of the oxidized product retained the C_{10} skeleton. They reported that oxidative attack was roughly distributed equally over the eight secondary carbon atoms. Another factor which may contribute to the instability especially of unsaturated esters during distillation, is the presence of traces of catalysts which promote conjugation and polymerization. These might take the form of acids resulting from oxidation or traces of metallic soaps (e.g. Cu or Ni) arising from the attack at high temperatures of fatty acids on metallic parts of the column (Murray, 1951).

IV. Relation of Fractional Distillation to other Separation Techniques

It is quite common that the mixture to be resolved is too complex for effective separation by fractional distillation alone. A mixture may contain several homologous series of acids, saturated, unsaturated or branched, and small

differences in boiling point of methyl esters of the same chain length makes separation by fractional distillation difficult or impossible. Fractional distillation effectively separates according to chain length or rather carbon family, and for mixtures containing more than one series of acids, is best used for this purpose in combination with another technique which separates according to type. The series may be broadly separated before distillation or the distilled fractions, collected according to chain length may be subsequently treated. The former procedure involves fewer operations but to make distillation the first step may be advisable or necessary when a wide range of chain lengths are present in the mixture or, again, when it is desired to deal with large quantities.

Separation methods based on solubility differences are widely used to separate saturated from unsaturated acids before fractional distillation of the methyl esters. The low temperature crystallization methods developed by BROWN and co-workers (BROWN and FOREMAN, 1944) have become well known as routine procedures, and have largely displaced the older method based on the solubility of the lead salts. The reader is referred to the chapter on this subject by BROWN and KOLB in this volume. The lead salt-ether method was used by ANDERSON and co-workers (see ANDERSON, 1939) for the separation of saturated straight chain and branched acids occurring in the tubercle bacillus, but a reinvestigation of the method by CASON and SUMRELL (1951) for the same purpose has shown that it is not applicable to branched acids generally. SHORLAND and co-workers (*loc. cit.*) have used low temperature crystallization methods in conjunction with fractional distillation and chromatography for the isolation of branched-chain fatty acids from various fats. In general, separations based on solubility are not very effective for the broad separation of series of acids when a number of chain lengths are present.

Chromatographic methods of separation recently reviewed by HOLMAN (1952) have a wide application to the separation of fatty materials according to type, and are capable in specific cases of high resolving power. The method most conveniently used in conjunction with fractional disillation is that of elution chromatography usually on columns of silica gel or alumina. Elution of compounds differing considerably in adsorptive properties may be made by a succession of solvents of increasing power of elution (the "eluotropic series" of TRAPPE, 1940). Displacement and carrier displacement chromatography offer great possibilities for the efficient separation of acids according to type. CLAESSON (1946) reported that separation of groups of normal saturated, unsaturated, and branched chain acids could be effected on specially prepared silica gel by displacement chromatography, while HOLMAN (1952) cites many examples of the separation of various types of acids of the same chain length by displacement and carrier displacement methods on carbon. Several recent investigations have used chromatography followed by fractional distillation to achieve difficult separations. HORN and HOUGEN (1953) separated a group of 1 : 2 diols from the main bulk of a wool wax non-saponifiable fraction by chromatography and then separated them by the fractional distillation of their acetates. MURRAY and SCHOENFELD (1953) used elution chromatography to

separate methyl esters of normal acids from those of hydroxy acids, and resolved the former series by amplified distillation. Almost pure samples of the methyl esters of arachidonic, eicosapentaenoic and doscosapentaenoic acids have been isolated from beef adrenal lipids by the combination of chromatography and fractional distillation (HERB, RIEMENSCHNEIDER, and DONALDSON, 1951; HERB, WITNAUER, and RIEMENSCHNEIDER, 1951).

The discovery by BENGEN (1940, 1951) of the formation of crystalline complexes of urea and linear molecules has made possible the separation of molecules according to their shape, or more strictly by their departure from linearity. This method of separation has been reviewed by KOBE and DOMASK (1952), TRUTER (1953) and by SCHLENK in a chapter in Volume 2 of this series. Since it separates according to shape it seems well adapted for combined use with fractional distillation which fractionates according to molecular size. SCHLENK and HOLMAN (1950) have shown that saturated and unsaturated acids and their methyl esters can be separated by preferential complex formation of the more linear saturated components, and by this means, followed by fractional distillation, easily prepared a methyl oleate of high purity. VON RUDLOFF (1951) was able to separate the aliphatic alcohols of wool wax cleanly from the predominating cyclic alcohols (cholesterol and triterpene alcohols) and submitted them to fractional distillation. Monomethyl branched acids and alcohols, such as the iso- and ante-iso-compounds, form urea adducts, but when esterified with a branched group so that the molecule is branched at both ends they fail to do so. This is the basis of a method of separation of monomethylated acids and monomethylated alcohols from straight chain and other compounds which has been described by WEITKAMP and BOWMAN (1952a (acids); 1952b (alcohols)). It has proved of value for the separation of the normal and branched alcohols of wool wax (MURRAY and SCHOENFELD, unpublished work).

AHRENS and CRAIG (1952) have investigated the separation of the higher fatty acids by the method of countercurrent solvent—solvent distribution using a 220 cell machine described by CRAIG *et al.* (1951). Separation of the four saturated acids C_{12} to C_{18} was easily accomplished as was also that of the unsaturated acids: oleic, linoleic and linolenic. However, when the resolution was attempted of mixtures of several series differing in degree of unsaturation, overlapping occurred. These workers proposed a combination of countercurrent liquid-liquid extraction with fractional distillation for the resolution of such mixtures. However, a more complete, if not total resolution, could be achieved by fractionation into material of equal chain length by amplified distillation, followed by the complete separation of saturated, mono-, di- and tri-unsaturated acids according to their method. DUTTON has reviewed the application of countercurrent distribution to lipid separations in a chapter in Volume 2 of this series.

V. CONCLUSIONS

It has been stated in a recent review (DEUEL and ALFIN-SLATER, 1952) that "newer methods for the separation of fatty acids are constantly being developed

and are to a great extent replacing the technique of fractional distillation of the methyl esters." It is considered that this statement does not do justice to the true position. The desire for methods to replace fractional distillation for this purpose has probably been due in the first place to the need, especially for workers in biological fields, to work on as small a scale as possible. This has led to the development of several chromatographic procedures for the separation of fatty acids on a milligramme scale. Special mention must be made of the elegant gas-liquid partition method of JAMES and MARTIN (1952) which shows an extraordinary high resolution of homologous fatty acids. However, chromatographic methods are still rather empirical and, as pointed out by HOLMAN (1952) are at present research tools rather than routine methods. No doubt they will have many applications to fat analysis, but it is not at all obvious that they will replace ester distillation when sufficient material is available.

Any attempt at an assessment of fractional distillation in relation to the newer methods of separation must take into account a number of factors. (i) Fractional distillation is dependent only on the properties of the molecules being separated, namely, their vapour pressures. If differences exist then separation is theoretically possible. Because of its fundamental nature its application is universal and reproducible. (ii) The progress of simple fractional distillation can be followed by boiling point, which also provides a means of identification. (iii) A wide range of chain lengths up to C_{30} can be fractionated with only changes in operating pressure. (iv) The use of amplified distillation permits charge of esters as small as 1 gm or less and enables almost complete separation of chain lengths differing by two carbons. (v) It is operated on a scale convenient for preparative work and for the identification of isolated substances by the classical methods.

Fractional distillation at low pressure as applied to the separation of fatty acids and related compounds in recent years has greatly increased in its resolving power and has been successfully used with increasingly smaller samples. Apparatus has been developed which has allowed it to be used with materials of progressively greater molecular weight, and the limits have still not been reached. Many separations hitherto difficult or impossible may now be easily achieved by its combined use with other separation techniques.

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8

FORMATION OF ANIMAL FATS

F. B. Shorland

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8

FORMATION OF ANIMAL FATS

*F. B. Shorland**

I. Introduction

Fats, as isolated from animal tissues, contain a complex mixture of fatty substances or lipids. In adipose tissues, true fats or triglycerides form perhaps not less than 99 per cent of the lipids present. On the other hand, in certain other tissues, such as liver or muscle, the proportions of phospholipids may exceed those of the triglycerides, and in addition, the extracted lipids contain substantial proportions of unsaponifiable constituents, particularly cholesterol. Within a given species the proportions of triglycerides vary considerably, being affected, for example, by the plane of nutrition, while the proportions of cholesterol and of phospholipids remain more or less constant. To distinguish between these two groups of lipids, Terroine[1] has suggested the terms "l'élément variable" and "l'élément constant." In this review it is proposed to limit the discussion to consideration of triglycerides and to refer to phospholipids merely to elucidate problems connected with triglycerides.

The amount of fat formed in the body seems to depend more on the caloric intake than on the nature of the dietary constituents. Well fattened animals may be produced from diets consisting almost entirely of proteins or of carbohydrates, or the diet may contain as much as 50 per cent fat. While fat formation in animals may occur endogenously without dietary fat except for traces of essential fatty acids, such as linoleic acid, nevertheless, from what follows, it will be apparent that the dietary fat when present often has a profound effect on the composition of the depot fat.

Animal tissues differ widely in fat content. Whereas in adipose tissues, such as the perenephric fat of pigs, the percentage fat may rise to 95 per cent,[2] in muscle tissues of certain fish such as the New Zealand ling (*Genypterus blacodes*)[3] or in the *longissimus dorsi* muscle of the rabbit[4] the level of fat (triglyceride) may fall to a fraction of 1 per cent. For unknown reasons the location of the fat deposits in the body varies considerably in different animals. In wild rabbits and horses there may be almost negligible quantities of fat in the abdominal cavity and not more than traces of subcutaneous fat. In pigs, sheep and oxen, on the other hand, there are usually extensive deposits in the abdominal cavity as well as layers of subcutaneous fat amounting in some instances to several inches in thickness. The subcutaneous fat of marine mammals, particularly whales, is usually even thicker, and in addition, in the sperm whale, there is a

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liquid wax deposition in the cavities of the head. In some fish such as South African jacopever (*Sebastichthys capensis*)[5] or the New Zealand groper (*Polyprion oxygeneios*)[6] the fat is diffuse, but in other fish such as the North Sea haddock (*Gadus aeglefinus*)[7] or the New Zealand ling (*Genypterus blacodes*)[3] the fat is concentrated almost entirely in the liver.

HAMMOND[8] has shown that as the animal grows there is a definite order in which the organs and different tissues develop. At first bone is the fastest growing tissue, then muscle overtakes the bone, and lastly fat develops. Normal age changes can, however, be considerably affected by the plane of nutrition. A high plane of nutrition hastens the normal age changes while a low plane of nutrition and slow growth delay them. At birth, farm animals have little or no visible fat; further growth involves changing a carcase that is almost devoid of fat into one that may contain four or five times as much fat as protein. Humans, however, differ from most other species in that the new born typically contains 16 per cent fat, and this amount is normally not exceeded in the adult.[9]

In fish it has been found that following spawning the fat reserves of the liver may be depleted while vitamin A, cholesterol and phospholipids remain more or less unaffected (*cf.* SHORLAND).[6] Preceding migration the fat reserves of fresh-water eels hitherto concentrated in the tail have been shown to move forward into the trunk, head and ovaries to provide a more uniform distribution.[10] Many investigators have confirmed the observation made by NAUMANN as early as 1822[11] that the beginning of the spring migration of birds is associated with a rich deposition of fat, but the data obtained have generally been of a semi-quantitative nature. ODUM and PERKINSON,[12] however, from an exact study of the seasonal variations in the subcutaneous, peritoneal and total body fat of white-throated sparrows, concluded that an individual bird would migrate only when fat deposition was completed. Changes in fat metabolism are also associated with egg laying. FLOCK *et al.*[13] found in geese during the egg laying season a marked increase in blood lipids and a spontaneous development of fatty livers.

It has become increasingly clear that adipose tissue is a tissue with a special structure and with a special type of cell which has other functions besides those of fat storage. Fat cells are controlled by the nervous system and supplied with blood capillaries. They contain enzyme systems which are capable of desaturating higher fatty acids and which convert into fat the glycogen that occasionally accumulates.[14] The concept of the adipose tissue as a more or less static reserve of energy is no longer supported but has given way to the concept of a dynamic state introduced by SCHOENHEIMER[15] arising from his studies with labelled fats.

It is intended here to trace the changes that take place in the dietary fat during the intraluminar phase of its intestinal absorption and its passage through the intestinal wall into the fatty depots. Consideration will then be given to the endogenous fat and its mechanism of synthesis and the interconversions that take place between various fatty acids. The complex question of glyceride structure cannot be dealt with in detail in this article, but the

factors which are responsible for the production of depot fats of a given fatty acid composition will be discussed.

II. Exogenous Fat

The dietary fat in its passage through the alimentary tract is usually mostly absorbed. It is generally agreed that this absorption occurs in the epithelial cells (villi) of the small intestine, and that most of the fat so absorbed passes via the lymph into the systemic blood. The exact mechanism of fat absorption is still somewhat uncertain, but owing to the development of techniques using isotopically labelled fats, and to improved methods for collecting lymph, many obscurities are gradually being clarified.

The reader is referred to the chapter by Bergström and Borgström in this volume, in which this subject is treated.

A. Digestibility of fats

Experiments with dogs,[16] humans,[17], [18], [19], [20] rats,[21], [22], [23] guinea pigs,[23] and farm animals[24] show that provided the dietary fat melts below body temperature it is usually absorbed to the extent of at least 90 per cent. This high level of absorption can be maintained even when the diet is rich in fat. Thus, Cook and Thompson[22] reported 92 per cent absorption for rats fed a diet containing 16·6 per cent fat. High melting point fats, however, are poorly absorbed. For example, Arnshink[16] found a digestibility coefficient of only 11·4 per cent for tristearin (m.p. 60°C) fed to dogs. According to Mattil and Higgins[25] the digestibility of fat containing stearic acid is poor and not closely related to the melting point, but is affected by the glyceride structure. A mixture containing two parts of tristearin and one part of triolein, for example, showed a digestibility coefficient of only 39·4 per cent as compared with 57 per cent for distearomonoolein. Hoagland and Snider[26] were also unable to find a consistent relationship betwen melting point and digestibility or to correlate digestibility with percentage of saturated acid or of linoleic acid. Bernhard[27] found that ethyl behenate (m.p. 50–51°) in a normal diet was absorbed by rats to the extent of 35–37 per cent and that admixture with olive oil did not appreciably increase the digestibility.

Apart from melting point considerations the absorption of fats may be affected by their chemical constitution. Thus rape seed oil showed a digestibility coefficient of 77–82 per cent,[28] probably on account of its high content of erucic acid.[27] Differences in digestibility coefficient of fats may be found among different animals. For example, castor oil which contains about 90 per cent ricinoleic acid is poorly absorbed by humans unless administered with a carrier glyceride such as olive oil,[29] but is well utilized by sheep, guinea pigs and rabbits.[30] Experiments with guinea pigs in which 6 per cent oleic acid or its *trans* isomer elaidic acid were included in the diet showed digestibility coefficients of 92–97 per cent and 44–69 per cent respectively. On the other hand, rats utilized both of these acids with equal facility.[30]

There appears to be little definite information as to the fate of fats fed to

ruminants. The products of digestion of carbohydrates and proteins arising from bacterial fermentation in the rumen are lower fatty acids, particularly acetic acid; there are indications that fats may also be broken down in the rumen as certain bacteria at least have been shown to oxidize fatty acids.[31] Moreover, fats of pasture-fed ruminants are almost entirely devoid of linolenic acid, the main constituent of grass and clover lipids.[32] REISER, however, considers that the relative absence of linolenic acid in the depot fats of pasture-fed ruminants might be explained by the fact that bacteria in the rumen hydrogenate linolenic acid.[33]

Thus it becomes clear from the differences in digestibility coefficients of different fatty acids that the composition of the dietary fat is modified during the process of absorption by the selective rejection of certain fatty acids. In ruminants it appears that this modification is even more extensive due to metabolism and, perhaps, hydrogenation of the ingested fat, so that the components presented for absorption bear little or no resemblance to those in the dietary fat.

B. Fat absorption

(1) *Intraluminar phase.* In the upper part of the small intestine the *p*H of the intestinal contents is normally about 6·5, which is somewhat below the optimum value for pancreatic lipolysis. Nevertheless, it is found that some hydrolysis of the triglycerides does occur and that mono-, di- and triglycerides as well as free fatty acids are present.[34], [35], [36] According to MATTSON *et al.*[37] the hydrolysis of the triglycerides is selective, the monoglycerides being present in the β form, while the diglycerides have the $\alpha\beta$ configuration. DESNUELLE and CONSTANTIN[38] found that the relative proportions of the products of the hydrolysis of triglycerides during the digestion were markedly affected by the presence of Ca ++ and bile salts. The rate and extent of the hydrolysis of ingested triglycerides also depends on their composition. Glycerides of long chain fatty acids, such as those typically present in natural fats, are slowly and incompletely hydrolyzed while glycerides of short chain fatty acids such as tributyrin, undergo rapid and complete hydrolysis.[39]

BORGSTRÖM[40] found that the hydrolysis of fats by pancreatic lipase was reversible. For example, 1-^{14}C stearic acid dissolved in corn oil and administered to rats yielded in the intestinal lumen glycerides with 16·9–51·5 per cent of the radioactivity of the fed mixture. The free fatty acids recombined in the intestinal lumen with mono- and diglycerides but not with free glycerol.

(2) *Intracellular phase.* According to FRAZER *et al.*[41] the fat in the upper part of the small intestine is present as negatively charged particles of less than 0·5μ diameter. Under the acid conditions (*p*H 6·5) in this part of the alimentary tract they found that the only possible combination which would yield a stable emulsion is free fatty acid/bile salt/monoglyceride. BERNHARD *et al.*,[42] however, found that D labelled fat fed to dogs in which bile was absent was still absorbed to the extent of 50–80 per cent, in place of the normal 95 per cent or more absorption. SEARLE and ANNEGERS[43] showed that in the absence of bile

the addition of an emulsifier did not restore the efficiency of fat absorption. It is thus seen that while bile is not completely necessary for fat absorption it has some significance in this connection other than its emulsifying properties.

The mechanism whereby the fat passes through the intestinal wall has been the subject of considerable discussion. Some fifteen years ago it was commonly accepted in accordance with the Pflüger-Verzar Hypothesis that complete hydrolysis of fats was an essential prerequisite for fat absorption.[44] This view can no longer be sustained in the light of the evidence already discussed pointing to the nearly quantitative absorption of fats under conditions of incomplete hydrolysis. In more recent years Frazer's Partition Theory has attracted considerable attention. As originally postulated,[45] the triglycerides are only partially hydrolyzed in the intestinal lumen. Owing to the hydrotropic action of the bile salts the free fatty acids thus liberated are absorbed into the blood capillaries of the intestine along with other water soluble substances, and pass to the liver via the portal vein. On the other hand, the glycerides are absorbed in particulate form and pass via the lymphatic system to the systemic circulation to be deposited in the fatty depots. The feasibility of such particulate absorption has been demonstrated by BAKER[46], [47] who has shown that during active fat absorption the fine canals which pierce the outer border of the intestinal cells are filled with stainable material. The evidence for the Partition Hypothesis was based mainly on chylomicron (glyceride particle) counts in the systemic and portal blood and on histological observations after feeding olive oil or oleic acid.[48] Further chylomicrograph studies by TIDWELL[49] did not confirm the earlier results of Frazer, but showed that free fatty acids followed the same lymphatic route as did the triglycerides. The reliability of the chylomicron and of the histological techniques has been questioned by REISER and BRYSON.[50] More recently BLOOM *et al.*[51] showed that the absorbed palmitic-1-^{14}C acid could be recovered to the extent of 92 per cent in the lymph, while other workers[52], [53], [54] obtained similar results for pentadecanoic, stearic and palmitic acids. In some cases these results have been interpreted as being contrary to the Partition Hypothesis but FRAZER[55] considers this to be erroneous as the acids had been fed in a glyceride vehicle. This objection, however, cannot be sustained, as experiments[56], [57], [58] using free fatty acids without carrier glycerides suggest that as for the corresponding glycerides the main pathway is via the lymph, the main point of difference being the slower absorption of the free-fatty acids as compared with the glycerides.[56], [57] Further, the presence of carrier glycerides has been shown to increase the rate of absorption of methyl oleate which by itself failed to emulsify in the small intestine.[29] The Partition Hypothesis was based originally on the partition of the fatty substances between the portal and lymphatic route, according to whether they existed as fatty acids or as neutral glycerides. Gradually, however, there has emerged a differentiation based on the physical properties, such as water solubility or otherwise of the forms of fat presented for absorption.[55], [59], [60] In this way the Partition Hypothesis explains the increasing tendency for fatty acids to follow the portal pathway

with decreasing chain length. Thus BLOOM *et al.*[61] found that on feeding 1-^{14}C labelled fatty acid to rats the following percentages of labelled carbon based on the amount of acid absorbed were recoverable from the lymph:-stearic acid 84–85 per cent, myristic acid 59–82 per cent, lauric acid 15–55 per cent and decanoic acid 7–19 per cent. Further evidence that decanoic acid passes into the blood capillaries was obtained by KIYASU *et al.*[62] who found that after feeding decanoic-1^{14}C acid there was greater activity in the fatty acids of the plasma of the portal vein than in the plasma of the inferior vena cava. These results do not preclude the possibility that decanoic acid is first broken down to acetate in the intestinal cell and then transmitted as a glyceride of a higher fatty acid. It is well known that lower fatty acids are not found in more than trace amounts in the depot fat even when present in substantial amounts in the diet. However, there is no definite evidence that the higher fatty acids are modified during absorption. Ingested labelled -1^{14}C pentadecanoic, palmitic and stearic acids have been recovered almost quantitatively from the lymph[52] while D labelled palmitic and stearic acids fed to rats, as such, or as glycerides, showed no evidence of desaturation in the intestinal lumen or in the intestinal cells.[63], [64]

Since the classical experiment of MUNK and ROSENSTEIN[65] in which ingested erucic acid appeared in the lymph as trierucin, it has been apparent that free fatty acids are transformed in the intestinal cell into triglycerides. It is also clear that the fatty acids present in forms other than triglycerides, such as, for example, ethyl esters,[66], [67], [68] amyl or cetyl[65] and mannite esters[67] when fed are not found in the lymph as esters, but as triglycerides. These results seem to indicate the extraordinary ability of the organism to modify fatty substances so that the fatty acids in lymph are present mainly as triglycerides. The ability of lipid fractions to interchange their fatty acid radicals has been further demonstrated by BORGSTRÖM.[69] Rats were fed three different fat mixtures: (A) corn oil interesterified with ^{14}C stearic acid and mixed with cholesterol, (B) ^{14}C stearic acid dissolved in corn oil and mixed with cholesterol, and (C) corn oil with cholesterol ester of ^{14}C stearic acid. The results of the investigation are summarized in Table 1.

Table 1. Interesterification of 1-^{14}C *stearic acid during intestinal absorption (Adapted from* BORGSTRÖM[69]*)*

State of administered 1-^{14}C *stearic acid*	*% total lymph fatty acids present as*		
	Cholesterol ester	*Glyceride*	*Phospholipid*
A. As glyceride	2·30 ± 0·27 (2·32 ± 0·42)*	87·8 ± 0·7 (88·4 ± 1·10)	9·82 ± 0·82 (9·24 ± 0·91)
B. As free acid	2·18 ± 0·22 (2·33 ± 0·33)	87·0 ± 0·6 (84·7 ± 0·89)	10·8 ± 0·55 (12·0 ± 0·66)
C. As cholesterol ester	2·46 ± 0·46 (2·86 ± 0·92)	86·0 ± 0·5 (82·7 ± 2·00)	11·5 ± 0·85 (14·3 ± 2·17)

* Figures in brackets indicate per cent activity of total fatty acids present.

Though there are significant differences between the activities of fatty acids in the glycerides and phospholipids (see Table 1) these are small, and the results indicate that interesterification is almost complete during the passage of the lipid mixture from the intestinal lumen to the lymph. As BORGSTRÖM has already shown that there is a partial interchange in the lumen,[40] further interchange would seem to occur in the intestinal cell.

Various workers have sought to elucidate the changes occurring during absorption by feeding labelled fats and analyzing the lymph lipids. REISER *et al.*,[70] for example, fed synthetic glycerides in which glycerol was labelled with ^{14}C and fatty acids with conjugated bonds. From analysis of the lymph they concluded that 25–45 per cent of the glycerides were completely hydrolyzed during absorption while the remainder was hydrolyzed to monoglycerides. Similar results have been obtained by BERGSTRÖM *et al.*[53] by BERNHARD *et al.*,[71] and by FAVARGER and COLLET.[72] The value of these techniques to measure the changes that occur during passage through the intestinal wall is restricted by the observation of BORGSTRÖM,[40] mentioned above, that some ester interchange occurs already in the intestinal lumen. There is, however, general support for the view that free glycerol[70] arising from hydrolysis of ingested fat or glycerol if fed[71] does not participate in the resynthesis of lymph glycerides but follows an independent pathway. According to REISER *et al.*[70] half of the lymph phospholipids is formed from endogenous glycerol and ingested free fatty acids, and the remainder from ingested glycerides.

General consideration of the results of various investigators thus indicates that the *glyceride structure* of the dietary fat is unlikely to remain intact during the intraluminar and intracellular phases of fat absorption. GARTON and DUNCAN[72a] have shown, however, that when pigs are fed a diet containing unusually high proportions (50 per cent by weight) of fat as equal parts of lard and cod liver oil, the triglycerides are absorbed and deposited without apparent change of structure. With regard to the *fatty acids* normally present in natural fats it appears that they remain unchanged during transference from the intestine to the lymph. The main aspect of fat absorption, apart from the question of the low digestibility coefficient of certain acids, is the interchange of fatty acid radicals. In whatever form a given mixture of fatty acids is present in the intestine, the fatty acids in the lymph will be distributed according to approximately the same pattern between the glycerides, phospholipids and cholesterol esters of the lymph.

C. Some factors affecting intestinal absorption

During fat absorption it has been observed that the fatty acids[73], [74], [75] and the phosphorus[76], [77] of the phospholipids of the intestinal mucosa undergo constant change. Although SINCLAIR[73] has postulated that phospholipids of the intestinal mucosa are intermediates in the synthesis of glycerides, other workers have considered that the rate of turnover of the phospholipids is insufficient for these substances to play a major role in this connection. The

recent work of BERGSTRÖM *et al.*,[78] however, shows that whereas 1-^{14}C stearic acid was rapidly and extensively incorporated into the intestinal phospholipids, 1-^{14}C myristic and palmitic acids were incorporated to a much lesser extent. Thus, the extent of the incorporation of ingested fatty acids into the intestinal mucosa phospholipids is dependent on their composition. The exact role of phospholipids in the resynthesis of glycerides is still not clear, but it would appear that the intermediate phospholipid formation in the synthesis of glycerides has more significance for some fatty acids than for others.

Choline has been shown by various workers to increase the rate of fat absorption.[79], [80], [81] Choline appears to be necessary for normal bile production.[82] In addition, according to FRAZER,[60] when fat particles are present in the intestinal cell choline will promote their discharge into the corium of the villus and thence into the lacteals by a mechanism which is thought to be due to the pumping action of the villus.

D. Transport and deposition of ingested fat

The fatty acids from the ingested fat with the probable addition of those synthesized from the non-fatty constituents of the diet by the intestinal cell (*cf.* BERNHARD and BULLET[83]) appear in the lymph mainly as triglycerides (see Table 1). BERGSTRÖM *et al.*[78] found in their experiments, that over a period of four hours the amount of labelled fat in the lymph entering the systemic circulation of the rat, mainly as triglycerides, was about forty times larger than the amount originally present in the plasma, but radioactivity of the plasma glycerides did not rise to more than one-sixth that of the lymph. Apparently the lymph glycerides are present as emulsified fat and as such are readily removed from the blood during their passage through the various organs. On the other hand, the original plasma lipids, which are not visible under the microscope, are much more stable, being present presumably as lipoproteins. In this form they retain their slower turnover during fat absorption without much admixture with the ingested fat that is transported by the blood plasma to the various organs of the body. Intravenously injected fat emulsions behave similarly to ingested fat in being rapidly removed from the blood stream.[84], [85], [86] The rapid removal of lymph glycerides from the blood is consistent with the fact that the concentration of lipids in rat plasma remains relatively unaffected during fat absorption. In certain other species including humans and dogs, the removal of these lipids from the blood is not so effective, and the blood lipids may increase to twice their fasting values during fat absorption.[87], [88], [89] BERGSTRÖM *et al.*[78] found that under the experimental conditions used the amount of phospholipids entering the blood stream from the lymph was only about one-tenth that of the glycerides, and furthermore, the lymph phospholipids had a lower specific activity than the corresponding glycerides. Nevertheless, the plasma phospholipids showed a higher activity than the plasma glycerides, indicating that the lymph phospholipids are more extensively mixed in with plasma phospholipids than are the corresponding glyceride fractions. Although the fat emulsion removed from the blood was largely (80–90 per cent)

glyceride containing fatty acids with a relative specific activity of 90 per cent, it was found that in the organs the radioactivity in the phospholipids was greater than in the glycerides. The liver phospholipids, for example, showed a rapid transfer of labelled stearic acid from the glycerides, as did the muscle phospholipids. GOLDMAN *et al.*[90] on the basis of studies of the distribution of isotopically labelled fatty acids in phospholipids of normal and liverless dogs after intravenous injection of 1-^{14}C labelled palmitic acid concluded that the liver was the site of synthesis of the plasma phospholipids. The phospholipids of heart, skeletal muscle, kidney, small intestine and its mucosa and lung were synthesized in the tissues of these organs and were not concerned with the transport of fatty acids from one organ to another.

As in the case of the intestinal phospholipids[78] earlier discussed, it would be anticipated that fatty acids would distribute themselves differentially between the glycerides and phospholipids of the liver (*cf.* [91]) and other organs so that the results might vary according to the nature of the fat ingested. Thus, while some investigators have found the dietary fatty acids to be incorporated to a greater extent into the liver phospholipids than into the liver glycerides,[92] [93] others have found the reverse.[94], [95]

The observations of BERGSTRÖM *et al.*[78] on the selective incorporation of fatty acids into phospholipids have provided a mechanism for the differentiation of fatty acid composition of phospholipids as compared with triglycerides in the muscle and various organs of animals. The extensive observations by various investigators, especially on livers of various species of animals, show that phospholipids as compared with corresponding glycerides contain more stearic acid and C_{20}–C_{22} highly unsaturated acids, but less hexadecenoic acid.[96] This circumstance results in a general similarity in fatty acid composition of phospholipids from different species in contrast to the marked differences shown between their glyceride fatty acid composition.[97] Although it might appear that the selective incorporation of fatty acids into phospholipids as compared with glycerides is based entirely on molecular weight, further investigation shows that this is not so. For example, in pasture-fed horses SHORLAND *et al.*[98] have shown that whereas the dietary fats and depot fats contain linolenic and octadecadienoic acids in the ratio 4 : 1, the ratio in the phospholipids is approximately 0·1 : 1; elaidic acid when present in the diet is also selectively incorporated into the phospholipids.[99]

As the proportions of triglycerides in animals are usually considerably in excess of the phospholipids the interchange of fatty acid radicals between these two kinds of lipids probably will not have any marked effect on the composition of the glycerides in the fatty depots. Much of the ingested fatty acid is transferred directly to the fatty depots.[100] The mechanism whereby glycerides from the blood pass into the cells of fatty tissues and liver is obscure, as is the mechanism for the mobilization of fat from the fat depots to the liver and vice versa. It appears, however, that the mobilization may be affected by dietary factors, by nervous factors, by hormones, and possibly by varying metabolic demands.[60]

E. Some factors in the production of fatty livers

The accumulation of fat in the liver may arise from excessive amounts of fat in the diet, or from the excessive production of endogenous fat, as was observed by FLOCK *et al.*[13] when geese were vigorously fed on a carbohydrate-rich diet. Starvation also results in excessive fat deposition in the liver, as does, incidentally, exposure to carbon-tetrachloride vapours.[93] Certain substances, such as cystine[101], [102] and anterior pituitary extracts,[103] accelerate the deposition of fat in the liver while other substances (lipotropic agents) such as choline have the opposite effect.

BEST *et al.* in 1934[104] found that rats fed a diet rich in fat (40 per cent), but deficient in choline, produced fatty livers. The increased fat in such livers consisted entirely of neutral glycerides. It was not characteristic of the phospholipid fraction and bore no relationship to the amount of carcase fat.[105] As the omission of choline from the diet affected the rate of growth of rats and resulted in matted and untidy fur, BEST and HUNTSMAN[106] regarded choline as an accessory food factor. CHANNON *et al.*[105] observed that the degree of fatty infiltration in choline deficient diets varied with the nature of the fat. Comparing beef dripping, butter, olive oil, cod-liver oil, coconut oil and palm oil, they found the most intensely fatty livers were produced by butterfat, and the least by cod-liver oil. The degree of fatty infiltration was later shown to be related to the content of C_{14}–C_{18} saturated fatty acids in the diet,[107] increasing with decreasing chain length from C_{18}–C_{16}–C_{14}.[108] Oleic acid was found to be much less effective than palmitic acid in causing infiltration of liver fat.[109] Ethyl laurate was found to induce fatal myocarditis,[110] and ethyl caprylate occasional renal haemorrhage and necrosis.[108] Ethyl esters of fatty acids of less than 12 carbon atoms were not effective in producing severe fatty livers.[108]

Beside choline other naturally occurring lipotropic substances including betaine,[111] methionine,[101] inositol[112] and lipocaic[113] are known. A comparison of their particular properties with those of choline has no special bearing on the present discussion as the main consideration here is the source of the extra fat in fatty livers. Considerable light has been thrown on this problem by BARRETT *et al.*[93] who fed mice a diet containing 15–20 per cent deuterated linseed oil, and choline, until a reserve of deuterated depot fat had accumulated. Subsequent treatment involving starvation, injection with anterior pituitary extract, or exposure to carbon tetrachloride vapours induced fatty livers with extra fat of the same deuterium content as the depot fat. In each instance therefore it was deduced that the extra fat in the liver had resulted from transport of depot fat to that organ. On the other hand, feeding a carbohydrate-rich diet poor in lipotropic factors resulted in excess liver fat which, by comparison with depot fat, was poor in deuterium, and had, therefore, been produced endogenously in the liver from carbohydrate. STETTEN and SALCEDO[114] approached the problem of the source of extra fat in fatty livers by administering D_2O to animals and ascertaining from the deuterium content of the fat deposited, the amount of endogenous fat present. In this way they

confirmed the results of BARRETT *et al.*[93] in regard to the source of extra liver fat arising from injection of anterior pituitary substance or from fasting. In addition, they found that the fatty liver from cystine feeding arose from an increased synthesis of fat from carbohydrate which resulted in more newly synthesized fat both in the carcase and the liver. The extra fat in the liver thus originated, not from impaired transport, but from overloading with endogenous fat. They showed further that in the absence of choline, rats fed a carbohydrate-rich diet had a higher content of liver fat, most of which had been synthesized endogenously. In the control animals, however, there were more newly synthesized fatty acids in the depots than in the choline deficient animals. Choline they considered, facilitated transport of fatty acids from the liver to the depots.

It has been generally assumed that the choline exerts its lipotropic action by mobilizing the liver fatty acids in the form of plasma phospholipids.[118] However, the role of these compounds in the transport of fatty acids has been questioned. ZILVERSMIT *et al.*[116] found that choline stimulated lecithin turnover in the liver, but not in plasma, and they concluded that choline does not act by increasing transport of fat via plasma phospholipids, but rather by stimulating the utilization of fats within the liver itself. ARTOM[115] subsequently found that choline or some substance formed from choline *in vivo* stimulated the oxidation of fatty acids. Very marked differences were found in liver slices prepared from choline supplemented rats as compared with choline deficient animals. Thus it appears that choline may not, as was formerly supposed, facilitate the removal of liver fat to the depots, but acts by increasing the rate at which the surplus liver fat is used up. Though the experiments of BARRETT *et al.*[93] support the view that the fatty liver obtained by exposure of the animal to carbon tetrachloride vapours is due to the transport of depot fat to the liver, other experiments by WINTER[117] point to carbon tetrachloride poisoning resulting in a reduction in fatty acid utilization. The capacity of liver tissues to oxidize fatty acids may have, therefore, considerable significance in the development of fatty livers.

It thus seems that fatty livers may be produced either exogenously from the dietary fat or endogenously from carbohydrates and proteins. The extra fat in the liver has been shown to arise from the failure, owing to the absence of lipotropic agents, or to liver damage, to metabolize the fat received or produced by the liver in normal amounts, or it may arise from overloading of the liver with unusual supplies of fat synthesized in the liver or received from the depots.

III. ENDOGENOUS FAT

It has been known since the classical experiments of LAWES and GILBERT[118] carried out nearly 90 years ago, that animals fed a carbohydrate-rich diet deposited considerably more fat than was ingested. As the amount of protein in the diet was insufficient to provide for the deposited fat it was inferred that the extra fat in the animal must have been synthesized from the dietary carbohydrate. More recently, LONGENECKER,[119] fed carbohydrate-rich or protein-rich fat-free diets to rats depleted of their fat reserves, and established that

fats of similar composition may be formed with equal facility from the dietary carbohydrate or protein.

The mechanism for the conversion in the body of carbohydrates and proteins to fat has been a subject of continued study, and although the precise details are still unknown, by use of isotopic tracers a great deal of light has been shed on the problem. In particular there is now very little doubt that fatty acids are synthesized from small molecules (two carbon atoms) in the form of acetate originating from carbohydrate, protein, or from the breakdown of the fat itself. It is proposed to cover briefly the earlier experiments involving the administration of labelled compounds to rats and to consider in more detail the later experiments carried out by the British workers, FOLLEY and POPJAK on the mammary gland tissues.

A. Evidence for the synthesis of liver and body fat from "acetate"

β-oxidation (degradation of fatty acids involving successive removal of acetate) is perhaps amongst the most firmly established theories of biochemistry. The converse has, however, only recently been established. In 1944 RITTENBERG and BLOCH,[120], [121] found that after the administration of CD_3 $^{13}COOH$ to mice and rats the fatty acids in the liver and carcase contained ^{13}C and D indicating that acetate could be converted to fat in the animal. Some typical results are given in Table 2.

Table 2. Isotope concentrations in lipids of mice fed labelled acetate
RITTENBERG *and* BLOCH [121]

(*The values are given in atom per cent excess*)

	Carcase		*Liver*	
	^{13}C	*D*	^{13}C	*D*
Total fatty acids	0·081	0·13	0·103	0·32
Saturated fatty acids	0·101	0·24	0·160	0·42
Carboxyl carbon of saturated fatty acids	0·179	—	0·290	—
Azelaic acid from "oleic"	0·090	0·14		
Perlargonic acid from "oleic"	0·071	0·15		
Cholesterol	0·047	0·24	0·087	0·67

The presence of ^{13}C and D in the total fatty acids after feeding CD_3 $^{13}COOH$ (*cf.* Table 2) indicates that both carbon atoms are employed in the synthesis of some components of the total fatty acid mixture. From the concentration of ^{13}C in the pelargonic and azelaic acids derived from oleic acid and the presence of twice the concentration of ^{13}C in the carboxyl carbon as the average of all the carbon atoms in the total fatty acids (*cf.* Table 2), RITTENBERG and BLOCH[121] expressed the view that the fatty acids had been formed by the successive condensation of acetate molecules. At first it was thought that acetoacetate was an intermediary in this condensation as the biological condensation of acetate to form acetoacetate had been previously well established

both *in vivo* and *in vitro*.[122], [123] Later studies by ZABIN and BLOCH,[124] however, indicated that acetoacetate is not an intermediary in the formation of fatty acids from acetate.

ZABIN[125] showed that stearic acid isolated after incubation of rat liver slices with carboxyl labelled acetate, contained almost all of the isotopic carbon in the carboxyl position. The finding demonstrates that chain elongation occurs by the addition of two carbon atoms to the carboxyl end of palmitic acid.

Subsequently ANKER[126] fed 1-^{14}C myristic acid to rats and degraded the palmitic acid isolated from the carcase fats. As a result of such degradation it was estimated that the specific activities of the carbon atoms in the palmitic acid were distributed as follows:

$$\underset{0{\cdot}5}{CH_3}\cdot\underset{1{\cdot}5}{CH_2}\cdot\underset{23}{(CH_2)_{11}}\cdot\underset{54\text{–}59}{CH_2}\cdot\underset{1{\cdot}4}{CH_2}\cdot\underset{10\text{–}14{\cdot}5}{COOH}$$

The values given are expressed as a percentage of the total activity of palmitic acid. The distribution of isotope indicates that the palmitic acid is formed by condensation of the carboxyl group of myristic acid and acetic acid.

ANKER[126] also showed that the fed 1-^{14}C myristate was partly converted to myristoleic and oleic acids. The isotopic distribution of the carbon atoms in hydrogenated oleic acid indicated that the formation of oleic acid probably occurs through the elongation of myristic acid to stearic acid followed by dehydrogenation.

From an examination of the fatty acids of mice that had been sacrificed four hours after injection with carboxyl labelled acetate, DAUBEN *et al.*[127] found that whereas palmitic acid was labelled more or less uniformly at every alternate carbon atom throughout the molecule, stearic acid showed a different distribution of isotope labelling, in that the specific activity of the carboxyl carbon was about three times that of the remaining alternate carbons in the molecule. This indicates the possibility that palmitic acid is mainly synthesized from two carbon units and only a small quantity of it is formed by the elongation of pre-existing higher fatty acid such as myristic. Stearic acid, on the other hand, may be formed to a considerable extent from the sixteen carbon moiety. Degradation of the unsaturated acids showed a similar pattern of isotope distribution and generally accords with the postulate of ANKER[126] that the formation of unsaturated acids may proceed by dehydrogenation of the corresponding saturated acid.

SWAN[129] found that the liver fatty acids of mice, isolated 15–100 minutes after intraperitoneal injection of 1-^{14}C acetate generally showed an even distribution of ^{14}C at alternate carbon atoms throughout the whole molecule. In the acids isolated from the shortest term experiments, however, there was an excess of ^{14}C in the carboxyl carbon as compared with the remaining alternate carbon atoms. SWAN suggests that this situation is consistent with a reaction between endogenous (unlabelled) fatty acids and the exogenous acetate at the beginning of the experiment. This uneven distribution of the isotopic labelling is supported by the results of DAUBEN *et al.*[127] for stearic acid mentioned above.

Although the evidence so far presented shows that acetate is an intermediate in fat formation, the possibility that other compounds such as pyruvate can participate directly without prior conversion to acetate has not been excluded. In experiments involving the administration of labelled acetate various investigators by comparing the specific activity of the acetate excreted as *p*-acetamido benzoic acid (*cf.* BERNHARD[128]) with that of the synthesized fatty acids have calculated that not less than one quarter of the carbon atoms in the endogenous fat comes from acetate.[124], [130], [131] However, this technique assumes that the isotopic concentration of the excreted acetyl groups represents the true isotopic concentration of the metabolic pool. Moreover, the 2-carbon units derived from pyruvic acid are a source of carbon for the higher fatty acids but do not contribute proportionately to the acetylation of foreign amines such as *p*-amino benzoic acid.[132] In later studies on the synthetic activity of the lactating mammary gland many different labelled intermediates, including pyruvate were fed, and it was concluded that acetate usually occupies the main, if not the exclusive, role as the immediate precursor in fat formation.[133]

In accepting acetate as the precursor in the production of endogenous fat there arose the difficulty that fatty acids below capric acid had not been shown to occur in animal depot fats. Recently, however, acetic and butyric acids have been detected in the liver glycerides of the rabbit[133] and a consecutive series of volatile fatty acids (C_2 to C_{10}) has now been shown to occur in the perenephric fats of the ox.[134] The most complete evidence of intermediates in the build up from acetate to higher fatty acids has been obtained from studies by British workers on the lactating mammary gland.[133] Here the products of synthesis are stabilized by their rapid removal into the milk ducts.

The recent work in the Fats Research Laboratory in connection with the isolation of uneven numbered fatty acids from ox fat,[134] normal heptadecanoic acid from hydrogenated mutton fat,[135] numerous branched chain acids from the milk and depot fats of ruminants, as well as from shark liver oil[97] lends support to the view that compounds other than acetate alone also participate to a limited extent in the formation of animal fats.

B. Investigations on milk fats as a contribution to the study of fatty acid synthesis

It is apparent that the milk fats could result from either (*a*) blood fat or (*b*) by synthesis in the mammary gland, or from both these sources. Cows receiving in their diet unusual fatty acids such as erucic, the highly unsaturated acids of cod-liver oil, or the lower saturated acids of coconut oil, incorporate these acids into the butterfat.[136], [137] It is therefore evident that a certain proportion at least of the milk fat will originate from the blood fat. HILDITCH[91], [138] has expressed the view that the milk fats of ruminants originate entirely from the blood fats, and that milk fats and depot fats are derived from a common precursor. He showed that the glycerides of ruminant milk fats, particularly of butterfat, and the corresponding depot fats differed from all other natural fats in containing considerably more fully saturated glycerides than would be expected if the fatty acids had been distributed amongst the glycerides according

to the principle of even distribution characteristic of all other natural fats. This situation, he considered, arose from the fact that although milk and depot fats were assembled originally on an even distribution pattern, the preformed oleo-glycerides in these fats were subsequently hydrogenated *in situ*. This resulted in considerably greater proportions of stearic acid than were found in the depot fats of other animals and to correspondingly increased proportions of fully saturated glycerides. In ruminant milk fats still further modification took place in the mammary gland by a process of ω-oxidation, whereby the stearic acid molecules arising from hydrogenation of the preformed oleo groups are degraded in the intact glyceride to give substantial amounts of C_4–C_{14} volatile fatty acids peculiar to these fats. In addition, some of the oleic acid groups in the intact glyceride are similarly degraded to give the C_{10}, C_{12} and $C_{14}\Delta^9$ acids present in minor proportions in milk fats.

In 1938 SMITH and DASTUR[139] found that during inanition the proportions of volatile acids in butterfat were greatly reduced and suggested that this finding was as much in harmony with the view that volatile fatty acids are intermediates in the synthesis of higher fatty acids from carbohydrates as with the view put forward by Hilditch. Subsequently other workers showed that the lactating ruminant udder *in vivo* had a respiratory quotient greater than unity, also indicating the possibility of synthesis of milk fat from carbohydrates.[140], [141]

Further advances were made by mammary gland tissue slice studies using acetate and glucose as substrates. FOLLEY and FRENCH found that mammary gland slices from lactating ruminants utilized acetate, but not glucose, with a respiratory quotient greater than unity.[142], [143], [144] Non-ruminants showed, on the other hand, a respiratory quotient greater than unity only in the presence of glucose, glucose and acetate, but not with acetate alone as substrate.[145] The utilization of acetate by mammary tissue slices of lactating ruminants had particular significance, because in ruminants the carbohydrates and proteins are broken down to lower volatile acids, particularly acetate, which is transported in the blood.[146]

It seems therefore that ruminants, in contrast to non-ruminants, have undergone a process of adaptation[147] so that the energy requirements for the conversion of acetate to fatty acids may be derived from acetate in place of glucose. Glucose, perhaps, has little importance in the synthesis of fat from carbohydrate in the ruminant udder. That mammary gland slices synthesize fat from small molecules has also been shown by the incorporation of ^{14}C from $CH_3\,{}^{14}COONa$ and of tritium T from THO into the fatty acids.[148]

Definite evidence for the synthetic activity of the mammary gland *in vivo* was first obtained by POPJAK *et al.*[149] After administration of 1-^{14}C acetate, it was found that the fat of the non-lactating mammae of pregnant rabbits contained isotopic carbon, particularly in the volatile acid fraction. This indicates that acetate was used chiefly for the synthesis of the short chain fatty acids.[150]

Other experiments demonstrated the synthesis of fat from acetate by the udder of the goat[151] and of the cow[152] and also by the isolated perfused

bovine udder. This shows that the synthesis of milk fat occurs in the udder itself and not in the liver or elsewhere.[153]

After intravenous injection of 5 mc of 1-^{14}C into a lactating goat, the specific activities of the milk fatty acids were examined at intervals during the next 48 hours, with the results shown in Table 3.

Table 3. Specific activities (1×10^{-3} μc ^{14}C per mg C) *of pure fatty acids obtained from glycerides of milk during various intervals after intravenous injection of* 5 mc *of* ^{14}C *as* $CH_3\,{}^{14}CO_2Na$ *into a lactating goat*

(Popjak, French, Hunter *and* Martin[154])

Fatty acid	*Period of experiment from which fatty acids were pooled*	
	0–12 *hour*	12–48 *hour*
Acetic . .	14·68	—
Butyric . .	11·45	0·350
Hexanoic . .	21·15	0·424
Octanoic . .	28·40	0·717
Decanoic . .	33·75	0·943
Lauric . .	23·80	1·080
Myristic . .	10·28	1·260
Palmitic . .	10·25	1·260
Stearic . .	0·49	0·063
Oleic . . .	0·31	0·063

At all stages it was found that the specific activity of the milk fat was several hundred times greater than that of the plasma,[155] indicating that apart from oleic and stearic acids (*vide infra*) synthesis of the milk fat had occurred in the mammary tissues. The results shown in Table 3 for individual fatty acids collected during the first 12 hours and at 12–48 hours after injection suggest a relationship between specific activity, chain length and the biochemical mechanism of fatty acid synthesis by the successive addition of acetate molecules to the carboxyl end.

The results in Table 4 show that butyric acid is synthesized by the condensation of two C_2 units of equal isotope content. On the other hand, the distribution of ^{14}C in hexanoic acid (*cf.* Table 4) shows that the "acetic" part contains 2·5 times as much isotope per labelled carbon atom as does the "butyric" part. This situation would arise if the butyric acid synthesized from acetate in the udder was being continuously diluted about 2·5 times by a non-isotopic C_4 compound. The dual origin of butyric acid in the udder may be represented thus:

$$CH_3C^{**}OOH \rightarrow CH_3C^{**}H_2CH_2C^{**}OOH + \text{non-isotopic } C_4 \text{ compound}$$

$$\text{e.g. butyric acid} \rightarrow CH_3C^{*}H_2CH_2C^{*}OOH$$

The synthesis of caproic acid may then be visualized by:

$$CH_3C^{*}H_2C^{*}OOH + CH_3C^{**}OOH \rightarrow CH_3C^{*}H_2CH_2C^{*}H_2CH_2C^{**}OOH$$

* Denotes the presence of labelled carbon atoms.

Table 4. Specific activities of individual carbon atoms of acetic, butyric, hexanoic and octanoic acids after injection of $CH_3{}^{14}COONa$ *(specified activities are expressed as* 1×10^{-3} *μc* ^{14}C *mg C) (Adapted from* POPJAK et al.[133], [154])

	Carbon atoms								Average of all c. atoms	
	1 (COOH)	2	3	4	5	6	7	8		
Acetic	23·6	0·00	—	—	—	—	—	—	11·8	1–48 hr after injection
Butyric	15·26	0·00	15·26	0·00	—	—	—	—	7·63	
Hexanoic	32·60	0·00	13·20	0·00	13·20	0·00	—	—	9·83	
Octanoic	92·60	0·00	59·6	0·00	30·00	0·00	30·00	—	28·4	1–12 hr after injection
Hexanoic (degraded from octanoic)									22·9	
									(21·15)*	
Butyric (degraded from octanoic)									15·0	
									(11·5)*	

* Natural acid.

The non-isotopic C_4 compound might be butyric acid itself or another C_4 compound such as β-hydroxybutyric acid, which is known to be present in the blood of lactating ruminants in sufficiently large amounts to be absorbed by the mammary gland.[156]

It is not known whether the mechanism of fat formation in the udder differs from that in the depot fat, apart from the stabilization of intermediates by rapid removal into the milk ducts. This removal may be responsible for the fact that as indicated from the specific activities of the fatty acids (*cf.* Table 3) the synthesis of fat in the udder stops at palmitic acid, but in depot fat it continues, yielding as the main constituent oleic acid. It is perhaps relevant that in the mice depot fat, as earlier indicated, palmitic acid is formed by the more or less simultaneous addition of C_2 units, but stearic acid is formed to a considerable extent by the addition of acetate to preformed C_{16} units.[127]

The comparative absence of radioactivity of oleic and stearic acids, and the marked radioactivity of the C_2–C_{16} acids indicated in Table 3, is consistent with the view that the former acids comprising 40 per cent of the total fatty acids of milk fats are derived from the blood fat, and the latter, by synthesis in the udder from acetate. The presence of marked labelling of the C_2–C_{16} acids, however, does not completely exclude the possibility that some of these acids found in milk fat could have been derived in part from the blood glycerides also. For example, when coconut cake containing lower saturated acids is fed to cows, the proportions of lauric and myristic acids increase in the butterfat.[136] Thus, while HILDITCH's view[91], [138] that milk fat is derived from the same precursor glycerides as the depot fat cannot be accepted completely, it is likely that somewhat more than 40 per cent (including oleic and stearic acids) is derived in this way. The decrease in the proportions of volatile acids during inanition[139] suggests that under these conditions a still greater part of the butterfat comes from the blood glycerides.

To explain the common pattern of the mixed glycerides in the milk and depot fats of ruminants, and at the same time to reconcile the syntheses of short chain fatty acids in milk fat from C_2 units, HILDITCH[157] suggested that the short chain fatty acids replaced oleic acid groups in the preformed triglycerides. The tendency for milk fat to contain less *c.* (22–25 per cent) palmitic acid than depot fats *c.* (27 per cent) would be consistent with some replacement of palmitic acid by lower saturated fatty acids in the mammary gland.

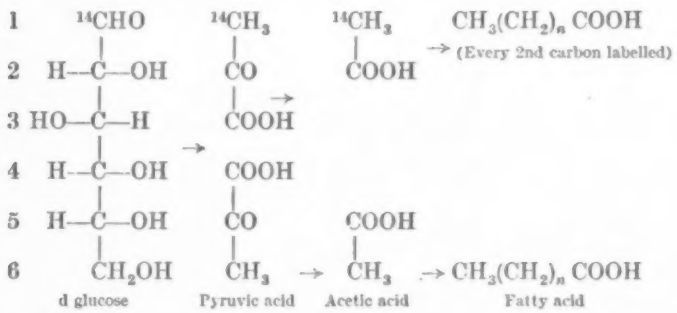
C. Fat synthesis from carbohydrate

Although GRAY *et al.*[158] have shown by adding labelled propionate to rumen fluid that this acid contributes to the formation of valeric acid, investigations have so far failed to establish the presence of an intermediate other than acetate for the biosynthesis of fatty acids in animals. In ruminants, the carbohydrate food is assimilated mainly as acetic acid, but in other species the endogenous metabolism of the animal must provide acetate by breaking down the larger molecules. It would seem that either carbohydrates or proteins may be used for this purpose. It is known that certain amino acids such as leucine[159]

and alanine[160] yield acetate in the animal, thus providing a means for the conversion of protein to fat. Other amino acids such as glutamine are well known as glycogen formers. As carbohydrates are converted in the animal to fats this provides a further pathway for the conversion of protein to fat.

The mechanism for the conversion of carbohydrate to fat has been made much clearer by the work of POPJAK *et al.*[161] in which the following compounds CH_3 $^{14}COOH$, $^{14}CH_3COOH$, $CH_3{\cdot}^{14}CO{\cdot}COOH$, $^{14}CH_3{\cdot}CO{\cdot}COOH$, ^{14}C-glucose and ^{14}C starch were administered to lactating rabbits. It was found that each of the test substances was equally effective as a source of carbon for the synthesis of fatty acids of milk fat and that the distribution of radioactivity in the water-insoluble volatile, water-soluble volatile and the non-volatile fatty acids was similar, irrespective of the compound fed. From these results it appears that the test compounds had first been broken down to acetate before utilization for fat synthesis. Further evidence in this connection was obtained from measurements of the specific activity of excreted "acetyl" C after administration of ^{14}C pyruvate, ^{14}C glucose and ^{14}C acetate respectively, using the *p*-amino-benzoic acid technique previously mentioned.[128]

Detailed examination of the distribution of the ^{14}C in isolated octanoic acid, also showed that the pyruvate was converted by decarboxylation to acetate before participation in the synthesis of fatty acids. Similarly, by feeding (1-^{14}C)-glucose, which is broken down in the glycolytic cycle in such a way that carbon atoms 1 and 6 become the β-carbon of pyruvate, it was possible to show that the results are consistent with the following mechanism for the conversion of carbohydrate to fat.



Besides acting as a source of acetate it has been shown by POPJAK *et al.*[162] that glucose or starch provides the glycerol required for the synthesis of triglycerides. It has been suggested by FOLLEY and FRENCH[163] that the stimulatory effect of glucose upon fat synthesis may be due to the increased availability of glycerol resulting from glucose breakdown. It appears further that the half-life of the fatty acids in milk glycerides of the lactating goat is 4 hr[118] as compared with 3 hr for glycerol.[162] The glycerol is metabolized by the body faster than the fatty acids. POPJAK *et al.*[161] from general considerations emphasize that the conversion of carbohydrate to fat requires the formation of glycerol as well as fatty acids for the synthesis of triglycerides.

(1) *Effect of vitamins of the B group.*—Until recently considerable emphasis has been placed on the role of vitamins of the B group in the synthesis of endogenous fat. McHenry and Gavin[164], [165], [166] considered that fat synthesis from carbohydrates did not occur unless thiamine was administered though the synthesis was augmented by the addition of riboflavin and pantothenic acid. Incidentally, they found that pyridoxine was needed for the conversion of protein to fat, which they considered took place through intermediary carbohydrate formation.[167] Guggenheim and Olsen[168] studied the effects of thiamine, pantothenic acid, biotin and choline deficiencies in young rats. They fed diets containing $CH_3{}^{14}COONa$ and found that although the total incorporation of radioactive carbon in the liver fatty acids varied as a result of changes in the absolute amount there was no effect on the specific activity of the fatty acids. This indicated that in these deficiencies lipogenesis *per se* is not altered. Boxer and Stetten[169] attributed the failure of the synthesis and deposition of fatty acids in thiamine deficient rats to diminished food intake rather than to any specific action of thiamine. By examination of the evidence of other investigators, and on the results of his own work, Gruber considers that there is no indication that thiamine deficiency causes impairment of fat synthesis from carbohydrate.[170], [171]

(2) *The influence of hormones.*—Insulin has been shown to stimulate fat synthesis in liver slices,[172], [173], [174] and in lactating mammary gland slices of non-ruminants provided glucose is present.[175], [176], [177] In ruminants, such as sheep, however, insulin was without effect on the lactating udder slices.[175] Because ruminants use acetate as the preferred energy-yielding substrate, while non-ruminants utilize carbohydrate, Folley[178] has suggested that insulin stimulates fat synthesis through its effect on carbohydrate for energy production.

Brady *et al.*[179] have shown that pretreatment of rats with cortisone decreases the ability of their liver slices to incorporate 1-^{14}C acetate into the fatty acids. By both respiration and isotope incorporation studies Balmain *et al.*[180] have demonstrated opposite *in vitro* effects and mutual antagonism of insulin and cortisone on lipogenesis by mammary gland slices from a lactating rat.

Brady and Gurin[181] have shown that liver slices of pancreatectomized cats and of alloxanized rats are relatively ineffective in converting acetate to fatty acid. In the case of the alloxanized animals the addition of insulin or glucose to the incubating medium failed to restore the lipogenic activity of the liver slices. Hypophysectomy[179] was found to stimulate hepatic lipogenesis in cats, but injection of the purified hormone or of cortisone inhibited the process.

(3) *Plane of nutrition.*—A low plane of nutrition has been shown by many workers to restrict very considerably the lipogenesis in liver slices. For example, Masoro *et al.*[182] found in rats on a diet containing 60 per cent dextrose and 22 per cent casein, the ability of liver slices to convert ^{14}C-labelled glucose to fatty acids was reduced to one tenth after the animals had been starved for 24 hr. The removal of carbohydrate from the diet also resulted in an

extraordinary reduction in hepatic lipogenesis. It was concluded that dietary carbohydrate is essential for the maintenance of the capacity of hepatic tissue to convert glucose to fatty acids. The results of LYON *et al.*[183] confirm the marked effect of carbohydrate on lipogenesis. In rats fasted for 18 hr the administration of 5 gm of glucose raised the fatty acid ^{14}C recoveries from a level of 2–7 per cent to as high as 40 per cent in liver slices to which had been added ^{14}C acetate.

D. Extrahepatic synthesis of fats

It was formerly considered that the formation of endogenous fat took place exclusively in the liver (*cf.* LONGENECKER[184]). While the importance of this organ in fat synthesis has been generally indicated,[90], [185], [186], [187] there has accumulated a great deal of evidence to show that many other tissues besides those of the liver possess lipogenic activity. The most outstanding example in this connection is the mammary tissue, which, as previously indicated, in lactating animals may be more active than the liver.

The first definite indications of extrahepatic lipogenesis were obtained by WAELSCH *et al.*[188] in 1940, using as indicator deuterium administered as D_2O in the drinking water. They showed that fatty acids are rapidly deposited in rat brain during myelination, but were unable to establish conclusively that the adult rat synthesizes fat in tissues other than those of the liver. The relatively large concentrations of deuterium in the fatty acid fractions of the intestine, however, suggested that this organ might also actively participate in endogenous fat synthesis.[189] Subsequently MASORO *et al.*[190] showed the appearance of ^{14}C-labelled fatty acids after injection of ^{14}C-labelled glucose in liverless animals whose intestinal tracts had been removed. CHERNICK *et al.*[187] found that surviving slices of rat kidney and of diaphragm converted ^{14}C-labelled glucose into fatty acids.

The synthesis of fatty acids in the liver and extrahepatic tissues of the rabbit foetus has been demonstrated by the fact that these tissues incorporated *in vivo* the administered deuterium as D_2O or the ^{14}C from ^{14}C-labelled acetate to a greater extent than in the maternal liver.

As the foetal liver fatty acids showed a higher concentration of ^{14}C but lower concentration of D as compared with the foetal extrahepatic tissues POPJAK and BEECKMANS[150] suggested that the fat precursors in the foetal liver and extrahepatic tissues may not be identical.

By use of D_2O and particularly by repeated intravenous injection of $CH_3\,^{14}COONa$ the same workers[191] established that in the adult rabbit extrahepatic synthesis occurred in the whole of the gastrointestinal tract as well as in the lung tissues. The concentrations of ^{14}C in the fatty acids of the lung and the intestine were respectively 30–45 times and 4–6 times that of the liver fatty acids. It was found that in the liver the specific activities of the fatty acids in the phospholipid and glyceride fractions were similar. In the intestine and particularly in the lung the specific activities of the phospholipid fractions were higher than those of the corresponding glycerides. These results, according to POPJAK and BEECKMANS[191] were consistent with the view that phospholipids

were primarily concerned with the synthesis of fatty acids. In the liver there was a rapid equilibrium between the phospholipid and glyceride fatty acids, but in the intestines and lung equilibrium was only slowly established.

From the correlation between the appearance of glycogen in adipose tissue and fat desposition TUERKISHER and WERTHEIMER[192] suggested that adipose tissue itself is capable of converting carbohydrates into fat. This has since been confirmed by the fact that adipose tissue containing glycogen exhibits a respiratory quotient exceeding 1·0[193], [194] and also by the fact that adipose tissue incubated *in vivo* with serum enriched with deuterium oxide contains deuterated fatty acids.[195] It thus seems that most tissues of the body including fatty tissues participate in the synthesis of fat.

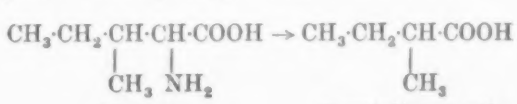
E. The occurrence of branched-chain fatty acids in animal fats

The fatty acids present in animal fats are almost exclusively straight-chained and with an even number of carbon atoms. One notable exception is isovaleric acid which is found as a major constituent in the fats of the dolphin and the porpoise.[196], [197]

Fatty acids which are branched, and which contain either an odd or an even number of carbon atoms, are known to occur in the non-glyceride lipids of certain bacteria and of wool grease (*cf.* HILDITCH[91]) and recent work by HANSEN and SHORLAND[198] revealed the presence of branched-chain acids in trace amounts in butterfat. Continuation of this work has shown that trace amounts of branched-chain fatty acids varying from C_{15} to C_{20} are also present in certain animal depot fats (*cf.* Table 5). Of the latter, some contain an even number of carbon atoms and some an odd number, while the number of side chains has been shown to vary from one to four. Lower molecular weight volatile acids (odd and even) C_2–C_{10} have also been shown to occur in the steam distillate from the fatty acids of ox perenephric fat.[134]

The occurrence of a C_{20} saturated acid with 3 or 4 branches cannot readily be conceived as originating by the successive condensation of acetate molecules. On the other hand, there appears to be no reason why the iso or anteiso acids should not be derived mainly from acetate, provided suitable end groups could be supplied from some other source. A possibility in this connection is afforded by the deamination of certain amino acids by the rumen bacteria. EL-SHAZLY[213] showed that some of the volatile acids in the rumen, including acetic, propionic, butyric, isobutyric and branched C_5 acids originated from protein breakdown. It is these branched-chain acids which are characteristic products of the breakdown of protein in the rumen under physiological conditions.

A possible mechanism for the endogenous synthesis of the anteiso acids isolated from mutton tallow or ox tallow would involve the deamination of L-isoleucine to yield 2L-methyl butyric acid.



L-isoleucine — 2L methyl butyric acid

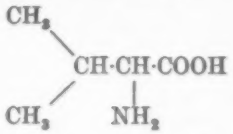
Table 5. The branched-chain fatty acids of animal fats

Source	Number of carbon atoms	M.p. 0°C	Number of side CH_3 groups	Position of CH_3 groups	Long spacings A°		Specific rotation in chloroform
					This acid	Corresponding straight chain acid	
Butterfat	C15[199]	52·0°	1	Iso	30·06	34·2[809]	
	C15[199]	23·7	1	Anteiso	30·05	—	$[\alpha]_D^{16·5°}$ + 4·8°
	C17[200]	54·4	1	Iso ?	33·9	38·6[808]	
	C17[200]	39·8	1	Anteiso	33·1	—	?
	C18[201]	33·5	1	10-methyl	33·5	39·75[810]	?
	C20[202], [203]	liquid	3 or 4	?	?		$[\alpha]_D^{20°}$ + 1·12
Ox tallow	C17[204]	40·5°	1	Anteiso	33·5	38·6	?
Mutton tallow	C15[205]	50·2	1	Iso	30·05	34·2[806]	—
	C15[205]	27·0	1	Anteiso	30·1		$[\alpha]_D^{22°}$ + 3·7
	C17[206]	38·0	1	Anteiso	30·0		$[\alpha]_D^{23·2°}$ + 3·83
	C17[207]	− 5	2	?	?		
Shark liver oil	C18[202]	*c.*−50	2	?	?		$[\alpha]_D^{14°}$ + 2·3°

* Melting points of the corresponding straight chain acids are:

(*a*) C15, 52·4°[1218]
(*b*) C17, 59·2–60·5°[1211]
(*c*) C18, 69·6°[193]

By the successive addition of acetate, L-2-methyl butyric acid would give rise to the anteiso acids isolated. The fact that the methyl side chain of L-2-methyl butyric acid and of the anteiso acids are of similar configuration (*cf.* KLYNE)[214] is consistent with this view. In a similar manner leucine could be a precursor of the iso-acids. The recent isolation of a C_{14} iso acid[215] from butterfat suggests the possibility of an endogenous synthesis from valine:



which EL-SHAZLEY regards as the precursor of the isobutyric acid found in the rumen.

The exact proportions of branched-chain acids are difficult to determine, but the data so far obtained are consistent with the presence of not less than 1 per cent of these constituents. As the unsaponifiable matter of the fats examined was not in excess of 0·7 per cent and the main component of this fraction, cholesterol, was largely in the free form, it seems probable that the branched-chain acids are not adventitious constituents accompanying fats but are present as part of the glyceride molecule.

The occurrence of volatile uneven numbered fatty acids in beef perenephric fat[134] as well as *n*-heptadecanoic acid in hydrogenated mutton tallow[135] mentioned earlier, affords further evidence of the probable complexity of endogenous fat production involving the participation of units other than acetate.

IV. The Dynamic State of Animal Depot Fats

A. Utilization and replenishment of fat in the body

The concept of a dynamic state of animal fats has been used by SCHOENHEIMER[15] to convey the idea that fat depots are not, as hitherto supposed, inert masses to be drawn upon against starvation but that depot fats are in a state of constant utilization and replenishment even under conditions of starvation.[216]

The first definite evidence of the dynamic state of animal depot fats was obtained by SCHOENHEIMER and RITTENBERG[100] in 1935. They found that normal adult mice fed a fat-free diet to which 2 per cent linseed oil labelled with deuterium had been added incorporated within four days 44 per cent of the dietary fat. As the amount of depot fat remained constant, it was evident that the deuterium-labelled fat in the depots had replaced an equivalent weight of fat that had been mobilized or degraded. More recently PHIL *et al.*[130] found that the fatty acids in the liver and carcases of rats fed labelled acetate added to a fat-free diet showed half-life period of 1·9 and 18 days respectively. This may be compared with the milk fatty acids of the goat in which the activity after injection with ^{14}C acetate decayed with a half-life of about 4 hr[155] showing an extremely rapid rate of synthesis. The notion of a dynamic state

carries with it the necessity not only of rapid synthesis but also of breakdown of fat. It is now generally supposed that the oxidation of fat in animal tissues proceeds along KNOOP's suggestion of β-oxidation yielding acetate which may be further oxidized through the tricarboxylic acid cycle or may combine with another molecule to form acetoacetate. There have been numerous experiments to show that fat is rapidly metabolized in the body. For example, STANLEY and THANNHAUSER[217] found that 50–73 per cent of the iodinated fat administered orally to humans was degraded within 24 hr. LERNER *et al.*[218] showed that the palmitic acid labelled with ^{14}C at its sixth carbon atom when injected intravenously into rats yielded in 24 hr 35–39 per cent of the ^{14}C as CO_2. GOLDMAN *et al.*[219] found that whereas the intravenous injection of palmitic acid 1-^{14}C into normal dogs was followed by the expiration of 11 per cent of the ^{14}C in 7 hr, liverless dogs under similar conditions expired 4 per cent of the ^{14}C as CO_2, demonstrating the activity of the extrahepatic tissues for fatty acid oxidation. This is also supported by GEYER *et al.*[220] who has shown that carboxyl labelled trilaurin is metabolized by kidney, liver, spleen, heart, lung, brain and skeletal muscle tissue slices. Other investigations demonstrating extrahepatic breakdown of fatty acids include those of WERTHEIMER and BEN-TOR[221] who found that fatty acids of glucose-free serum decreased on incubation with rat diaphragm, while diaphragms from fasting rats equilibrated with glucose manifested theoretical respiratory quotients for fat oxidation.

Studies by CHAIKOFF *et al.*[222], [223] in which palmitic acids labelled with ^{14}C at their carboxyl, 6th and 11th carbon atoms were injected intravenously in the form of their triglycerides into fasted rats showed that the location of the label in the palmitic acid chain did not influence significantly the amount or the rate of elimination of $^{14}CO_2$. It appears, therefore, that once the process of breakdown is initiated in the intact animal, a palmitic acid molecule is disrupted in such a manner that all of its carbons are converted to CO_2 at about the same time. In the oxidation of stearic acid a portion is first converted to palmitic acid. This difference in behaviour on oxidation between palmitic and stearic acid is paralleled by the differences in their mode of synthesis from acetate observed by DAUBEN *et al.*[127]

In addition to the disappearance of fat by oxidation in the tissues, some of the fat is converted into glucose. This is particularly noticeable in diabetic rats in which STRISOWER *et al.*[224] record that at least 6–12 per cent of the injected palmitic acid -6-^{14}C was incorporated into glucose in 24 hr. Various workers[183], [225], [226] have shown a lack of correlation between lipogenesis and CO_2 elimination. For example, the former is affected considerably by the addition of glucose to the diet while the latter is not.

In recent years considerable information has accumulated indicating the importance of coenzyme A in the synthesis and degradation of fatty acids. LELOIR and MUÑOZ[227] described in 1944 the first cell-free preparations capable of fatty acid oxidation using particulate material from the guinea pig liver, but their activity was usually limited to the oxidation of butyrate. Subsequently LEHNINGER[228] found that the oxidative activity resided in the easily

Table 6. Interconversion of fatty acids in animal tissues as shown by isotopic labelling

A. Deuterium labelled fatty acids

Species	*Fatty acid administered*	*Tissue examined*	*Labelled fatty acids found*	*Reference*
Mice	Stearic	Carcase	Oleic	(SCHOENHEIMER and RITTENBERG,[238] 1936)
Mice	Oleic	Carcase	Saturated	(RITTENBERG and SCHOENHEIMER,[237] 1937)
Rats	Stearic	Carcase	Palmitic	(SCHOENHEIMER and RITTENBERG,[232] 1937)
Rats	Palmitic	Carcase	Palmitic, stearic, myristic, lauric and oleic	(STETTEN and SCHOENHEIMER,[239] 1940)
Rats	Lauric	Carcase	Lauric, myristic, palmitic and stearic	(KLEM,[240] 1943)
Rats	Myristic	Carcase	Myristic, palmitic	(KLEM,[240] 1943)
Rats	Behenic	Carcase	Stearic, palmitic, myristic and oleic	(BERNHARD and VISCHER,[241] 1946)

B. ^{14}C *or* ^{13}C*-labelled fatty acids*

Species	*Fatty acid administered*	*Tissue examined*	*Labelled fatty acids found*	*Reference*
Rats	Acetic	Carcase and liver	Total fatty acids	(RITTENBERG and BLOCH,[120] 1944)
Rats	Acetic	Carcase and liver	Total fatty acids, saturated acids and oleic acid	(RITTENBERG and BLOCH,[121] 1945)
Rats	Acetic	Liver slices	Stearic	(ZABIN,[122] 1951)
Goats	Acetic	(Milk fat)	Even numbered fatty acids C_2 to C_{16}	(POPJAK, FRENCH, HUNTER and MARTIN,[124] 1951)
Rats	Lauric 1-^{14}C	Carcase	C12, C16 and C18	(STEVENS and CHAIKOFF,[242] 1951)
Rats	Myristic 1-^{14}C	Carcase	C14, C16 and C18	(STEVENS and CHAIKOFF,[243] 1951)
Rats	Myristic 1-^{14}C	Carcase	Palmitic, C14 and C16 unsaturated acids	(ANKER,[126] 1952)
Mice	Acetic 1-^{14}C	Carcase	Palmitic and stearic	(DAUBEN *et al.*,[127] 1953)
Mice	Acetic	Liver	Total fatty acids	(EWAN,[128] 1952)

sedimented particulate fraction of rat-liver which, under appropriate conditions, oxidized higher fatty acids.[229], [230] Complete oxidase activity was later shown to be present in the mitochondria prepared from 8·5 per cent sucrose homogenates.[231]

The active intermediate in both acetoacetate and citrate formation has now been isolated by LYNEN *et al.*[232] and shown to be the acetyl-Co A derivative. The structure and synthesis of this compound have been described by NOVELLI[233] and the steps in the synthesis and degradation of fatty acids have been followed by LYNEN and collaborators[234] using spectrophotometric methods. It has been demonstrated that the first step in oxidation is the formation of an $\alpha\beta$-unsaturated acid, which, in turn, is converted to the corresponding hydroxy acid. The hydroxy acid is then oxidized to the keto acid and finally removed as an acetate unit.

LYNEN[234] and MAHLER[235] have given mechanisms for the above reactions. From consideration of these it is possible to see that if carboxyl labelled acetate were fed to animals, there would be a tendency for the carboxyl groups of fatty acids synthesized in the animal to be more heavily labelled than the remaining alternate carbon atoms.

B. The interconversion of fatty acids

Besides the synthesis and breakdown of fatty acids, the dynamic concept involves the interconversion of fatty acids In this connection it is convenient to consider separately the fatty acids which may be produced endogenously and the fatty acids which cannot be synthesized by the animal from non-fatty sources but which are derived entirely from the fatty constituents of the diet. In regard to the endogenously produced fatty acids, certain interconversions of fatty acids have already been referred to in previous discussions. More extensive data indicating the nature of these interconversions are collected together in Table 6. The evidence in general is based on the administration of labelled fatty acids followed by examination of the fats in the tissues. Isolation of fatty acids from the tissues with the same label as the administered fatty acid is taken as evidence of the origin of the tissue fatty acid from the administered fatty acid.

The data in Table 6 show that besides elongation or shortening of the fatty acid chain by the addition or removal of C_2 units, the organism is able to desaturate. This desaturation is limited to the introduction of a double bond in the Δ^9 position. Further evidence of the specificity is afforded by the work of APPEL *et al.*[243] on feeding synthetic fats containing a continuous range of saturated uneven-numbered fatty acids to goats. From the depot fat of the latter there was obtained a range (C_{11}–C_{19}) of unsaturated fatty acids. In each case it was found that the unsaturated fatty acid had the double bond in the Δ^9 position and had the configuration (*cis*) of natural oleic acid.

The evidence relating to the interconversion of fatty acids which has so far been described may be summarized thus:

Behenic (C22)
⇅
Stearic (C18) ⇌ Oleic (Δ^9 octadecenoic acid)
⇅
Palmitic (C16) ⇌ Palmitoleic (Δ^9 hexadecenoic acid)
⇅
Myristic (C14) ⇌ Myristoleic (Δ^9 tetradecenoic acid)
⇅
Lauric (C12)
⇅
Short chain fatty acids
⇅
Acetic

The relatively constant proportions of palmitic acid in animal fats noted by HILDITCH,[91] and the lack of marked change in the fatty acid composition of sheep[244] and pig depot[245] fats during inanition are consistent with the rapid interconversions of fatty acids. In this way on fat-low diets, the fatty acid composition is modified, giving fat characteristic of the species.

The complete range of even-numbered fatty acids from C_2–C_{18} acids is found only in milk fats, in which, as previously discussed, the metabolic products are stabilized by secretion into the milk ducts. In depot fats the proportions of fatty acids containing less than 10 carbon atoms are negligible. Fatty acids with less than 10 carbon atoms when ingested, are deposited mainly as palmitic and stearic acids.[246], [247], [248] It appears that these short chain acids are first fragmented into C_2 units which are then recombined to form long chain fatty acids.[174] The tendency in depot fats is to produce fatty acids of the C_{16} and C_{18} series. Thus BERNHARD and VISCHER[242] found that the behenic acid 1 1 as deuterio-behenic ethyl ester to rats was degraded to acids of 18 carbon atoms or less, and no arachidic acid was formed.

The rapid adjustment of fatty acid composition of depot fat does not seem to apply so readily to fatty acids which are not produced endogenously. For example, VISSCHER[249] has shown that 24 per cent of hendecanoic (C_{11}) acid may be stored in rat depot fat. APPEL *et al.*[243] found, however, that the glycerides of odd-numbered straight chain fatty acids fed to rats are subjected to some interconversion. Thus the fatty acid composition of the ingested fat differs considerably from that of the depot fat (see Table 7), the latter including unsaturated uneven numbered fatty acids not present in the diet.

LOVERN[250] has suggested that extensive hydrogenation or dehydrogenation of the fatty acids in fish may occur. Fish, however, appear to derive most, if not all, of their fat exogenously in contrast to the fats of mammals which are often mostly of endogenous origin.

In contrast to almost all other aquatic fats, which contain as major constituents C_{20} and C_{22} highly unsaturated fatty acids, the liver oil of the shark (*Scymnorhinus lichia*) contains no significant amounts of polyethylenic acids but only monoethylenic acids[251] indicating probable hydrogenation of the ingested fat. From a comparison between the seasonal changes in the composition of herring fat and those of the food fat, LOVERN[252] concluded that

hydrogenation and dehydrogenation occurs to a considerable extent in the depot fat of this species.

Although di- and polyethenoid fatty acids are not synthesized by the animal, nevertheless the absence of certain of these acids, notably linoleic ($\Delta^{9,\ 12}$ octadecadienoic) results in a fat deficiency disease, which is accompanied by an impaired growth, scaly skin, caudal necrosis and lesions in the kidney and urinary tract.[253]

Table 7. Percentage fatty acid composition (expressed as methyl esters) of body fat from rats fed on fat composed of odd-numbered fatty acids C_9–C_{19} (Appel et al.[243])

Chain length	*Fatty acids in diet*	*Fatty acids % in depot fat*	
		Saturated	*Unsaturated*
C9	3·85		
C10	—		
C11	5·94		
C12	—	1·50	0·11
C13	47·10	16·26	1·22
C14	—	3·29	0·31
C15	19·86	11·02	0·66
C16	—	7·19	7·79
C17	11·35	15·76	6·32
C18	—	1·59	22·20
C19	11·90	1·39	3·30

Arachidonic ($\Delta^{5,\ 8,\ 11,\ 14}$ eicosatetraenoic acid) has an activity similar to that of linoleic acid[254] while linolenic ($\Delta^{9,\ 12,\ 15}$ octadecatrienenoic acid) has somewhat lesser activity.[255]

Nunn and Smedley-MacLean[256] found that methyl linoleate when fed to rats maintained on a fat-free diet gave rise to fatty acids in the liver yielding polybromides corresponding to those of arachidonic acid. Methyl linolenate, on the other hand, resulted in the formation of acids which yielded polybromides corresponding to a mixture of arachidonic and docosapentaenoic acids. Reickehoff *et al.*[257] and Widmer and Holman[258] using the more sensitive alkali-isomerization technique (*cf.* Holman and Burr[259]) confirmed that rats fed on a fat-free diet when supplemented with linoleic acid produced arachidonic acid in the various tissues. Linolenic acid, however, was chiefly concerned with the formation of hexaenoic acid and to a lesser extent with pentaenoic acid. On a normal diet it was found that rats deposited polyunsaturated fatty acids in their tissues. In decreasing order, heart, liver, brain, kidney, blood and skeletal muscle were found to be active in this respect.[258]

Unsaturated fatty acid conversions in poultry have been studied by Reiser using alkali-isomerization techniques. It was shown that feeding linoleic acid to chicks on a fat-free ration gave rise in the carcase and organs to pentaenoic acid and possibly trienoic acids while linolenic acid produced dienoic, tetraenoic, pentaenoic and hexaenoic acids.[260]

In eggs, REISER demonstrated that on fat-free rations, supplementation with linoleic acid was followed by the appearance in the yolk lipids of dienoic, pentaenoic and possibly tetraenoic acids. Linolenic acid produced all polyunsaturated acids, having from 2–6 double bonds.[261] α-elaeostearic acid when included in the diet was deposited in the yolk along with newly synthesized dienoic acid, while conjugated linoleic acid, when fed, was similarly deposited at first, but the subsequently produced eggs contained a fatty acid similar to the *in vitro* oxidation product of linoleic acid in having a broad absorption band at 270 mμ.[262]

The interconversion of fatty acids in hens is thus seen not to be identical with that in rats and the situation is summarized in Fig. 1 below.

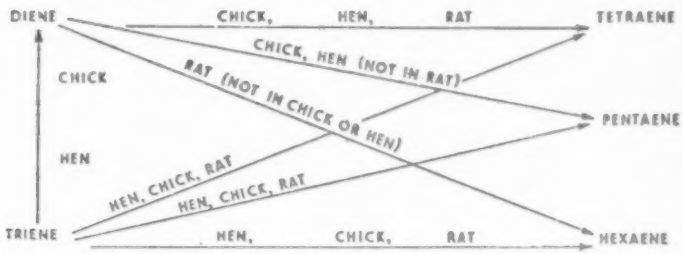


Fig. 1. Biological interconversion of polyethenoid fatty acids. (After REISER and HOLMAN[263].)

HOLMAN[264] found that the *trans* isomers of the linoleic and linolenic acids were much less active biologically than the naturally occurring *cis* forms. *Trans* linoleic acid was found to be not active in alleviating fat deficiency symptoms but was converted in the rat to a limited extent to tetraenoic and hexaenoic acids. *Trans* linolenic acid stimulated the formation of hexaenoic acid but promoted only moderate weight gain and fat synthesis. No polyethenoid acid formation occurred in animals receiving conjugated linoleic acid. The administration of this acid resulted in decreased weight and early death of the rats.

PRIVETT and HOLMAN have recently indicated by private communication that *cis*-9, *trans*-12-linoleic acid is also unable to relieve dermal symptoms or lessen water consumption of rats fed a fat deficient diet.

The steps involved on the biological conversion of linoleic and linolenic acids into polyunsaturated C_{20} and C_{22} acids have not been fully established. MEAD *et al.*,[265] however, found that labelled acetate adds to linoleate in the conversion to arachidonate in the rat, while KLENK and BONGARD[266] consider that the positions of the double bonds in the various C_{20} and C_{22} unsaturated acids isolated from brain lipids could best be explained by the addition of C_2 units at the carboxyl end of preformed oleic, linoleic and linolenic acids.

HOLMAN has recently reviewed[268a, 268b] in detail the work on essential fatty acid deficiency diseases.

WITTEN and HOLMAN[267] found that the biological conversion of linoleic to

arachidonic acid, and of linolenic to hexaenoic acid in the rat was considerably facilitated by pyridoxine, but benzoyl peroxide or tocopherol was inactive in this connection.[268] However, benzoyl peroxide greatly increased the conversion of linoleic to hexaenoic acid, and when fed with unsaturated esters promoted the formation of body fat. Thus the apparent toxic effect of benzoyl peroxide is reversed when fed with unsaturated fats.

V. Factors Determining the Fatty Acid Composition of Depot Fat

From what has already been considered it is apparent that depot fats are derived from two sources: (*a*) exogenous fat modified during absorption and transport and, (*b*) endogenous fat synthesized via acetate from the non-fatty constituents of the diet. The fatty acids after deposition (as triglycerides) are subject to further modification. Some fatty acid molecules will be converted to acetate and then reconverted to fat or transformed into carbohydrate or else broken down to carbon dioxide and water. Some saturated fatty acids will become unsaturated and vice versa.

There is evidence that the rapid turnover which is characteristic of the endogenous fat does not apply equally to the ingested fatty acids which cannot be synthesized by the animals. For example, Anderson and Mendel[269] showed that the iodine value of rat depot fat was raised following the feeding of soy bean oil containing linoleic and linolenic acids. The high iodine value thus produced, however, could not be markedly lowered when the soy bean oil diet was replaced by a high carbohydrate diet. Similarly, Longenecker[270] found that rats fed on corn oil diet after inanition with 24 per cent loss in weight, retained 29·8 per cent linoleic acid in their depot fats as compared with 32·3 per cent in the non-fasted animals. According to Kohl[271] when elaidic acid was fed to rats, to the extent of 40 per cent of the caloric intake, for three days, over thirty days were required before the reserves of elaidic acid were cleared from the body. Chevallier *et al.*[272] showed that polyunsaturated acids did not disappear from the bodies of rats on fasting for 140 hours when depletion of fat was well advanced.

From discussion in the section dealing with exogenous fat (see page 310) it will be apparent that the fatty acids after ingestion are transported in the blood mainly as triglycerides to the various tissues and organs. Here they undergo selective interchange with the fatty acids of the phospholipids.[78] Fig. 2 gives an approximate representation of the processes involved in the formation of fat in non-ruminants. As shown in the diagram, in the intestinal lumen carbohydrates are converted into glucose and other monosaccharides. Proteins, on the other hand, are hydrolyzed to amino acids. In ruminants, hemicelluloses, cellulose (which are practically indigestible in non-ruminants) and proteins are broken down by bacteria in the rumen to volatile fatty acids, particularly acetic acid. The formation of volatile acids in the rumen is followed by their passage into the blood for subsequent use as a source of energy and for the synthesis of fats, carbohydrates and cholesterol (*cf.* Elsden and Phillipson,[273] Huffman[274]). Thus in ruminants conversion of food to

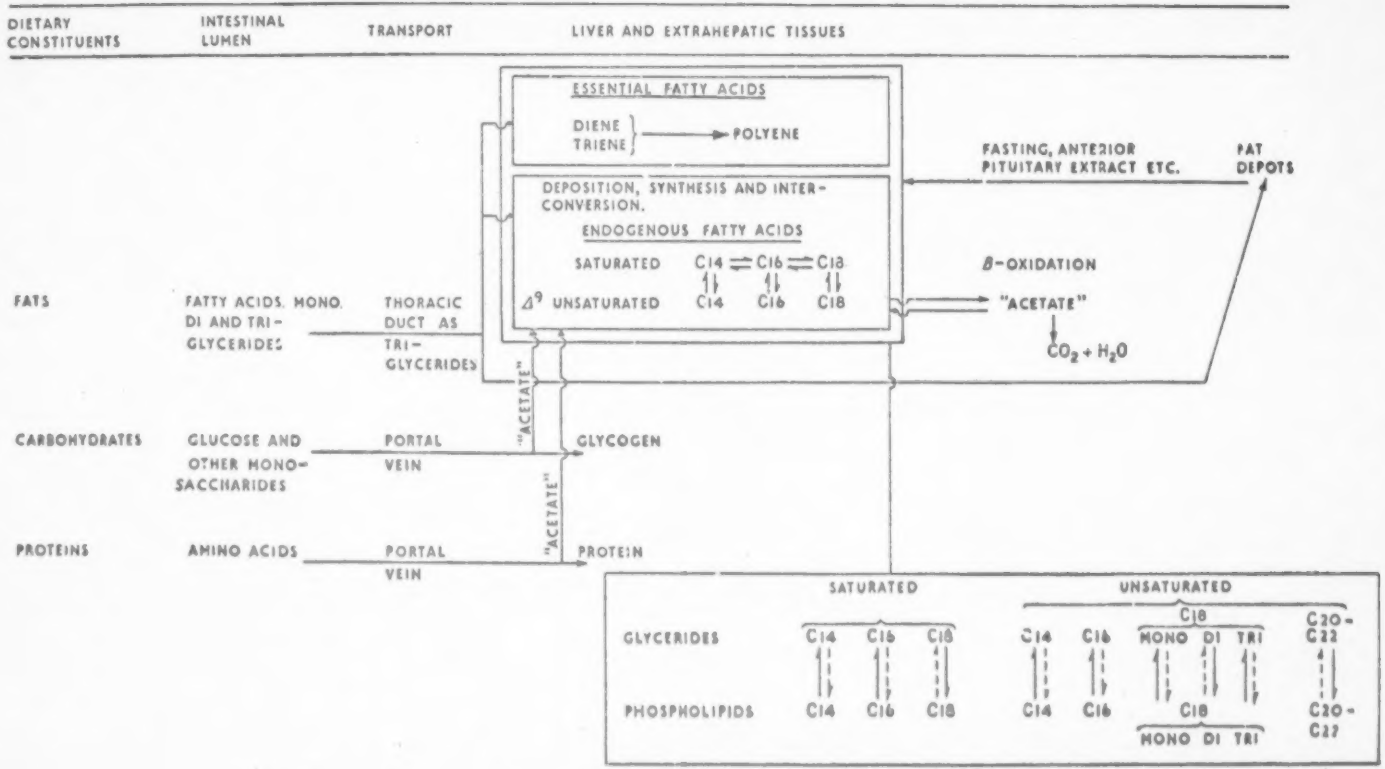


Fig. 2. Simplified diagrammatic representation of the stages involved in the formation of animal depot fats

acetate occurs in the alimentary tract whereas in non-ruminents glucose and amino acids are converted to acetate in the liver and other tissues after their passage through the intestinal wall.

A. Glyceride structure

Although Fig. 2 stresses the main point that depot fats of animals are the product of a mixture of exogenous and endogenous fats which are in a state of continual modification, it represents a considerable oversimplification of the true position. It does not take into account, for example, the intricacies of glyceride structure. Difficulties arise from the lack of precise methods for the investigation of glyceride structure. Nevertheless, during the past 25 years this subject has been studied extensively by HILDITCH who formulated the widely accepted rule of even distribution.[91] According to this principle the fatty acids are distributed as widely as possible among the glyceride molecules so that, for example, no fully saturated glycerides are formed until the content of saturated acids exceeds two thirds of the total. To account for the fact that in ruminant depot fats fully saturated glycerides are formed before the saturated acid content reaches 66·7 per cent. HILDITCH suggests that such fats have been assembled originally on an even distribution pattern, but modified subsequently by the hydrogenation of preformed oleoglycerides *in situ*. The problem of glyceride structure is of great importance and interest as illustrated by the recent work of QUIMBY *et al.*[275] which shows that lard consists largely of glycerides with palmitic acid in the β-position, whereas beef and mutton tallow contain palmitic acid mainly in the α-position. This probably accounts for some of the differences in the physical properties of tallows and lard. This work also confirms HILDITCH's views to some extent, in suggesting that the basic pattern of fatty acid distribution in animal fats is not based upon the law of probability.

Other theories of fatty acid distribution have been formulated by other investigators (*cf.* DEUEL[276]). LONGENECKER,[277] for example, is of the opinion that the distribution of fatty acids in animal fats occurs on the basis of the law of probability. More recently KARTHA[278], [279] assumed that the general pattern of glyceride structure of natural fats is based on the law of probability, but that when the melting point of a fully saturated glyceride would be unduly raised by the inclusion of a saturated acid of high molecular weight, the third saturated acid that would otherwise enter to form a fully saturated glyceride is replaced non-selectively by an unsaturated acid.

It is now proposed to consider so far as is possible in such a dynamic system, the separate effects of the endogenous and exogenous fats on the composition of the depot fat.

B. Fatty acid composition of endogenous fat

The fatty acid composition of the endogenous fat may be deduced approximately: (*a*) from the fatty acid composition of animals fed on a "fat-free" diet, (*b*) from the composition of fat from pasture fed ruminants which appears

Table 7. The fatty acid composition of endogenous fat
(Fatty acids mols %)

(a) Animals fed on a "fat-free" diet

Species	Diet	Saturated					Unsaturated				
		C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{16}	C_{18}	C_{18} ol.	C_{18} lin.	C_{20-22}
Rat[119] (Total fat)	Sucrose	—	3·1	26·7	3·6	0·4	0·9	15·6	47·2	2·2	0·3
	Casein	—	2·8	29·7	5·8	1·1	1·0	15·6	43·8	2·0	0·2
Pig[250] (Outer back)	Skim-milk	—	2·5	28·9	9·8	—	—	5·3	53·4	0·1	—
(Inner back)		—	3·1	29·2	11·9	—	—	5·9	49·3	0·5	0·1
Sheep[244] (External tissue)	Wheat straw	0·4	4·4	25·9	23·6	—	0·4	0·8	42·5	1·5	0·5
(Perinephric)		—	3·3	24·7	36·6	—	0·2	1·1	31·2	2·1	0·8

(b) Pasture-fed ruminants

Species	Saturated					Unsaturated				
	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{16}	C_{18}	C_{18} mono-αί-tri-ene		C_{20-22}
Ox[281] (Perinephric)	0·1	3·9	28·7	30·4	0·6	0·5	1·8	32·4	0·8	—	0·8
(Intermuscular)	—	4·0	27·5	17·1	0·4	1·0	4·2	43·2	1·0	—	1·6
(Intramuscular)	—	1·3	28·0	14·0	0·8	0·5	5·9	47·0	1·0	—	1·5
(Subcutaneous)	—	3·8	28·9	12·8	0·8	1·7	4·6	44·1	1·3	—	2·0
Ox[281] (Perinephric)	0·2	3·7	26·5	23·1	0·7	0·5	2·6	40·4	1·8	—	0·5
Sheep[282] (Total fatty tissues)	1·7	4·2	26·6	21·3	0·6	0·6	1·8	42·4	tr.	tr.	0·8
Deer[96] (Perinephric)	0·2	4·9	38·4	28·6	2·6	0·3	2·4	16·5	1·2	1·0	3·9

(c) Estimation of composition of endogenous fat of 200 lb bacon pigs from comparison of weights of fatty acids deposited with weights of fatty acids fed

Diet	Saturated			Unsaturated	
	C_{14}	C_{16}	C_{18}	C_{16}	C_{18}
Separated milk with addition of mixed meal (High plane of nutrition)*	—	35·9	20·1	3·4	40·6
Separated milk with addition of mixed meal (Low plane of nutrition)*	—	33·3	21·0	1·1	44·6
Skim-milk only† (Full fed)	1·8	29·5	10·9	4·9	52·9
Skim-milk + maize meal† (Full fed)	0·4	34·7	14·4	2·9	47·6

* Calculated from data of HILDITCH, LEA and PEDELTY.[203]
† Calculated from data of SHORLAND and DE LA MARE.[209]

to consist almost entirely of endogenous fat, or (c) by comparing the weights of individual fatty acids in the depot fats with those in the diet.

It is not possible to raise animals on completely fat-free diets. Traces of so-called essential fatty acids (linoleic, linolenic and arachidonic acids) are needed for growth as well as for the prevention of scaly skin and caudal necrosis. In addition it is difficult to prepare diets that have been completely freed from fat. The evidence in Table 7 generally supports the view that endogenous fat contains oleic, palmitic, stearic, hexadecenoic and myristic acids. The traces of C_{18} diene and of C_{20}–C_{22} unsaturated acids invariably present very probably originate from the diet, though the possibility of some synthesis cannot be excluded.[284], [285] It is apparent from Table 7 that endogenous fat varies with different species and with the site of deposition. From Tables 7a and 7b the following degree of variation is indicated.

	Mole %
Myristic	1·3–4·9
Palmitic	24·7–38·4
Stearic	3·6–36·6
Hexadecenoic	0·8–15·6
Oleic	16·5–53·4

The composition of the endogenous fat seems to be affected by various factors. For example, Bratzler *et al.*[286] fed a "fat-free" vitamin E low diet to weanling pigs for 75 days. Vitamin E supplementation was found to affect markedly the oleic acid content of the body fat. The control animals contained 63·7 per cent as compared with 68·7–73·9 per cent in the experimental animals with added vitamin E.

The dietary fat appears also to influence the nature of the endogenous fat. For example, rats fed on diets rich in palmitic acid deposit less than the normal proportions of this acid,[105] but the endogenous fats of pasture-fed rabbits and horses (see Table 9) contain a higher than normal content of palmitic acid (45·8 and 36·3 per cent respectively, calculated from linolenic acid content). Rats fed on diets with 3–5 per cent of fat normally deposit fat with 5–8 per cent of hexadecenoic acid,[91] but the proportions of this acid may be increased two or three times by withdrawing fat from the diet,[119] or possibly by means of a hypothalamic lesion to induce obesity.[287]

C. The influence of exogenous fat

There are many experiments to show that the dietary fat may profoundly influence the composition of the depot fat. In the extreme cases where the diet includes considerable proportions of fat, the fatty acid composition of the dietary fat and of the depot fat may become almost indistinguishable. Thus rats fed corn oil deposit fats containing similar proportions of fatty acids to those present in the diet[270] (*cf.* Table 8). Often, however, there are complicating factors so that the effect of the dietary fat is not so obvious as is

Table 8. Modification of the composition of depot fat by feeding dietary fat. Fatty acids (mols %)

Fat	*Diet*	*Saturated*						*Unsaturated*					
		Below C12	C12	C14	C16	C18	C20	C12	C14	C16	C18		C20
											ol.	*lin.*	
Rat (total fat)	Sucrose[110]	—	—	3·1	26·7	3·6	0·4	—	0·9	15·6	47·2	2·2	0·3
Rat (total fat)	Corn oil[370] (84% total calories)	—	—	0·7	12·2	2·9	0·2	—	—	6·9	44·8	32·3	—
Corn oil		—	—	1·7	11·0	2·9	—	—	—	1·6	48·8	34·0	—
Rat (total fat)	Coconut oil[288] (84% total calories)	0·7	31·8	18·1	18·6	2·7	0·6	0·6	1·5	4·1	20·1	1·2	—
Coconut oil		19·4	47·2	16·4	8·5	1·7	0·2	—	—	1·1	5·5	tr.	—

illustrated by the fatty acid composition of fats from rats fed on coconut oil.[288] Here there has been considerable deposition of the lower fatty acids present in the dietary fat as shown by Table 8, but the proportions of these acids in the dietary fat are not paralleled closely by those found in the depot fat. The lower saturated acids are evidently preferentially metabolized by the animal.

The first clear recognition of the role of dietary and of "synthetic" fat in the formation of animal depot-fat appears to be due to CALLOW.[289] In 1935 he suggested that the softness and iodine value of fats in different parts of the body of the same animal, or between the same parts of different animals fed on the same diets, were determined by the rate of growth. He explained his view as follows: "It appears probable that the faster the rate at which fat is deposited in fatty tissues, the more saturated the fat becomes. This would be expected in view of the fact that fats in such deposits can either be formed from fats and oils in the diet, or from carbohydrates by synthesis. Since the fats and oils in the diet are limited in amount, the rate of deposition of fat may be so great that a considerable proportion of the fat must be synthesized from carbohydrate. This leads to an increase in the saturation of the deposited fat, because fat synthesized from carbohydrate is relatively saturated, with an iodine number of 50 to 60, whereas fat formed from oils in the diet is relatively unsaturated, owing to the fact that the oils in the diet usually have iodine numbers of over 100."

This view has superseded the earlier temperature theory of HENRIQUES and HANSEN[290] by offering an explanation of the variations in iodine value along the back fat of pigs. It made it possible to predict the softening effect of dietary oils (containing linoleic acid) on pig fats under the usual conditions of farm management, and is in agreement with results of GARTON, HILDITCH and MEARA[291] for pigs that had been fed on a special diet containing 50 per cent of whale oil. In these cases the acids concerned, viz. linoleic and highly unsaturated C_{20}–C_{22} acids are not readily transformed or mobilized. On the other hand, for certain other fatty acids, including generally those that are built up from acetate in the body, CALLOW's view is no longer applicable because the acids concerned are metabolized or transformed and not subject to prolonged storage. Difficulties in accepting growth rate as the only factor in controlling the composition of depot fats are:

(*a*) Contrary to growth rate principles, pigs fed on skim milk (containing only traces of low-iodine-value fats) show a similar gradation in iodine value, both along the back fat and between the inner and outer back fat, to that when softening oils are present in the diet.[292]

(*b*) In the abdominal, gizzard and neck fats of the hen, studied by HILDITCH, JONES, and RHEAD,[293] though the fat tissues must have grown at different rates, and considerable proportions (about 20 per cent) of linoleic acid were present, no appreciable differences in fatty acid compositions were found.

(*c*) In some instances the fatty acids from the dietary fat are selectively redistributed between the different fatty depots.[98]

(*d*) The depot fats of pigs fed on buttermilk and copra do not conform to a simple mixture of "synthetic" fatty acids and of coconut oil, but to a modified mixture in which the higher-molecular-weight fatty acids of coconut oil have been stored preferentially to those of lower molecular weight.[280]

(*e*) Contrary to CALLOW's view, buttermilk- and copra-fed pigs, which grew quickly, as compared with those that grew slowly, showed a lower iodine value[292] and contained more of the dietary myristic and lauric acids.[280]

The relative absence from the depot fat of the lower fatty acids, even when present in quantity in the dietary fat, may be associated with their different pathway of absorption directly into the liver (*cf.* FRAZER[55]) instead of by the more usual route for fats.

Because of the different behaviour of different dietary fatty acids and apparent species differences, four types of depot fat may be distinguished:

(i) A mixture of unchanged endogenous and dietary fat, e.g. in pigs fed on a diet containing 50 per cent of whale oil.[291]

(ii) A mixture of endogenous fat and of dietary fat with the fatty acids unchanged in their proportions but interwoven to give new types of triglycerides, e.g. linolenic acid occurs in pasture mainly as dilinoleno-triglycerides,[98] but in pasture-fed horses as monolinoleno-triglycerides.[294]

(iii) A mixture of endogenous and dietary fat, the latter greatly modified in fatty acid composition, e.g. the depot fats of buttermilk- and copra-fed pigs.[280]

(iv) Endogenous fat almost entirely, the dietary fat having negligible effect, e.g. the depot fats of ruminants, which is discussed below.

From the evidence already considered the following factors appear to influence the composition of the depot fat: (*a*) the amount and composition of the dietary fat, (*b*) the rate of growth of the fatty tissues, and (*c*) differential distribution of the dietary fatty acids between the various fatty tissues.

D. The relationship between species and depot-fat composition

(1) *Fatty acid composition of the depot fats of pasture-fed animals*.—Besides the factors already discussed the species of animal influences the degree to which the dietary fat modifies the composition of the depot fat. This is illustrated by farm animals in New Zealand. As these are pasture-fed throughout the year, with only occasional supplementation with hay, it is possible, without a specially designed experiment, to compare the depot-fat composition of different animals on a diet that is reasonably uniform in the amount (about 3 per cent of the dry matter) and nature (mainly linolenic acid) of its fatty acids.[97] In Table 9 the results for the fatty acid composition of rye-grass, the main constituent of first-class pasture in New Zealand, have been selected as representing the dietary fat.

The influence of the dietary fat on the composition of the depot fat shows variations with different animal species (see Table 9). Whereas the rabbit, and to a lesser extent the horse, incorporate the C_{18} triene (linolenic) acid and the C_{18} diene (octadecadienoic) acid from the diet into their depot fats, the depot fats of the sheep and deer (ruminants), feeding in the same field, contain no

Table 9. Comparison between the fatty-acid composition of the dietary fat and of the depot fats of pasture-fed animals
Fatty acids (weight per cent)

Species	*Source of Fat*	*Saturated*					*Unsaturated*						
										C18			C20–22
		C12	C14	C16	C18	C20 *and above*	C12	C14	C16	*Mono-ene*	*Diene*	*Triene*	
Rye-grass[97]	Leaf (glycerides)	0·4	1·4	10·6	1·5	0·4	0·2	0·5	4·1	4·6	11·6	62·8	1·9
Wild rabbit[97]	Abdominal	—	1·6	22·1	6·4	0·3	—	0·4	4·4	12·7	7·9	42·4	1·3
Horse[96]	Total fatty tissues	—	2·4	29·7	4·3	0·2	—	1·4	6·5	32·5	3·8	16·1	3·1
Deer[97]	Perinephric	0·1	5·1	35·9	29·6	2·9	—	0·2	2·2	17·0	1·2	1·0	4·8
Ox[97]	Caul and kidney	—	2·7	27·8	21·6	—	—	0·3	2·5	42·5	0·5	0·3	1·8
Sheep[203]	Total fatty tissues	1·3	3·5	25·0	22·2	0·7	—	0·5	1·7	44·2	trace	trace	0·9

more than traces of these constituents. The theory that the composition of the depot fats of ruminants is not substantially affected by the composition of the dietary fat is also supported by the earlier work of THOMAS, CULBERTSON, and BEARD,[295] who showed that feeding liberal allowances of highly unsaturated oils to steer calves over a period of 260 days had no perceptible effect on the mean unsaturation of the body fat.

(2) *The occurrence of* trans *acids in ruminant fats*—Although the fats of pasture-fed ruminants contain *trans* acids (3·5–11·2 per cent) (*cf.* [296], [297], [298]) they are absent from those of non-ruminants unless supplied in the diet. It was concluded that ruminants possessed a mechanism for the synthesis of *trans* acids since the rumen contents of sheep contained these acids (*c.* 3 per cent of the total fatty acids) whereas their diet (pasture) gave no indication that they were present.[296] REISER[33] has shown that linolenic acid, the main fatty constituent of pasture, is hydrogenated by bacteria in the rumen. It is well known that hydrogenation of unsaturated fatty acids is accompanied by the formation of *trans* isomers, and HARTMAN *et al.*[296] therefore suggested that this process is responsible for their formation in ruminants. These facts serve further to differentiate between the fat metabolism of ruminants and non-ruminants.

VI. THEORY OF EVOLUTION AND FATTY ACID COMPOSITION

HILDITCH and LOVERN[299] in 1936 showed that many close parallelisms existed between the kind and proportions of fatty acids combined as triglycerides in the depots of animals and their place in the evolutionary scale of development.

Table 10. Component fatty acids (weight %) in the depot fats
(HILDITCH and LOVERN,[299] 1936)

	Saturated	*Unsaturated*			
	Palmitic C16	C16	C18	C20	C22
Fish, freshwater	13–15	*c.* 20	40–45	*c.* 12	0–5
Fish, marine	12–15	15–18	27–30	20–25	8–12
Whales	12–15	15–18	35–40	15–20	5–10
Frog	11	15	52	15	
Tortoise	14	9	65	7	
Lizard	18	10	56	5	
Domestic fowl	25–26	6–7	*c.* 60	0·5–1	
Rat	24–28	7–8	*c.* 60	0·3–0·5	
Pig*	25–29	2–3	50–65	0·3–1	
Ox*	27–30	2–3	40–50	0·2–0·5	

* Composition revised in accordance with more recent information (*cf.* HILDITCH,[93] 1947).

From Table 10 it is apparent that in passing from fats of aquatic animals to those of land mammals there is a marked simplification in fatty acid composition. Whereas the fats of aquatic animals contain invariably the saturated acids C_{14}, C_{16} and C_{18}, and C_{14}, C_{16}, C_{18}, C_{20} and C_{22} unsaturated acids with from one to six double bonds, the fats of the terrestrial mammals, as a rule, contain

mainly palmitic acid (25–30 per cent) and oleic (C_{18} unsaturated) with minor proportions of other acids. Sometimes as in the depot fats of pigs, sheep and oxen, considerable proportions (10–30 per cent) of stearic acid are also present.

The question has been raised by SHORLAND[300] as to whether the increasing simplicity in the composition of animal fats as one proceeds from the lower to the more highly organized forms of life, is due to the species themselves or to the fortuitous influence of the diet.

As already discussed, the depot fats of non-ruminants (in contrast to those of ruminants) show changes in fatty acid composition with changes in dietary fat. No natural foodstuff is entirely free from fat, and it follows therefore that the differences between the fats of pasture and those of plankton are likely to account in part for the differences in fatty acid composition between the depot fats of pasture-fed animals (*cf.* Table 9) such as rabbits and horses and those of aquatic species (*cf.* Table 10). For similar reasons aquatic mammals or birds deposit fats which resemble those of plankton in containing a whole range of fatty acids from C_{14}–C_{22} whereas land mammals or birds which feed on fats of relatively simpler composition, deposit simpler types of fats containing only three acids—oleic, palmitic and stearic—as the main components.[91]

Although the nature of the dietary fat thus exerts under most circumstances a considerable influence on the composition of the depot fat, nevertheless, in the course of evolution there appears to be discernible changes in the nature of fat metabolism of animals. From these changes it is possible to suggest subdivisions of animal species in regard to fat metabolism as follows:- (*a*) species that deposit dietary fat only, (*b*) species that deposit both exogenous and endogenous fat, and (*c*) species that deposit endogenous fat only. These subdivisions cannot be completely rigid, but they represent an attempt to use a biological rather than an empirical basis of classification.

A. Species that deposit exogenous fat only

At first the organism, as exemplified by fishes, is apparently unable to make fat from the non-fatty (protein) constituents of the diet,[250] but derives its fat from the dietary fat. The dietary fat is usually deposited in the tissues more or less unchanged, but in some instances the dietary fat is modified by hydrogenation as in the liver oil of the shark *Scymnorhinus lichia*. Here the polyethylenic acids characteristic of the C_{20}–C_{22} group in aquatic species have been replaced by monoethylenic acids, and there are produced, in addition, higher fatty alcohols combined as ethers with glycerol.[251] The interconvertibility of fatty alcohols and fatty acids in the rat has been shown by STETTEN and SCHOENHEIMER[239], [301] with the aid of deuterium. In other instances, as in the herring depot fats, there is evidence that certain groups of unsaturated acids in the diet have been dehydrogenated while others have been hydrogenated.[252] In certain fish such as the rat fish (*Anarrhicas lupus*) and the shark (*Scymnorhinus lichia*) elongation or shortening of the fatty acid carbon chain occurs. This results in a deviation from the normal marine type of fat.[250]

Other variations in the fatty acid composition of fish oils arise from the differential distribution of the fatty acids between the liver and the other fatty depots. For example, as indicated by RAPSON *et al.*[302] fish with their oil concentrated in the liver show normal fatty acid composition of their liver oils. Fish with a diffuse oil system in which the liver is not the main centre of deposition show an abnormal fatty acid composition in containing increased proportions of C_{18} and C_{18} unsaturated acids, but correspondingly diminished proportions of C_{20} and of C_{22} unsaturated acids. Their main fatty depots on the other hand may show a normal marine type fatty acid composition.[303] In all these changes referred to, however, the pattern of the fatty acid composition of the dietary fat is still discernible.

In special circumstances, when the nature of the dietary fat is changed, so, too, is the composition of the depot fat. Grass-fed carp, for example, deposit diene and triene acids,[304] characteristic of grass lipids, but which are not found in the fats of other aquatic species. Similarly, eels fed on fats which differ in fatty acid composition from those originally present in their depot fat, have been shown to modify their depot fat to resemble more closely that of the dietary fat.[305]

B. Species that deposit exogenous and endogenous fat

In this category are included amphibians, reptiles and non-ruminants which use carbohydrate and protein to produce endogenous fat. Such endogenous fat consists mainly of palmitic acid, usually *c.* 27–30 per cent and oleic acid with minor proportions of myristic and palmitoleic acid. The content of stearic acid is variable (usually less than 10 per cent), and is evidently dependent on the presence of hydrogenases. The addition of vitamin E to a fat-free diet devoid of this vitamin was found to increase the proportions of oleic acid in the body fat at the expense of stearic acid.[236]

The composition of the depot fat will now depend on the nature and amount of the dietary fat and on the rate of growth as suggested by CALLOW,[289] provided the diet, as in coconut oil, does not contain lower fatty acids that are rapidly metabolized.

The fatty acid composition of marine mammals shows many unusual features, as in baleen whales (*cf.* LOVERN[306]). There appears to be less C_{20} and C_{22} unsaturated acids than are present in typical marine fish liver oils, suggesting that the depot fats are no longer derived entirely from dietary fat but include endogenous fat. Hydrogenation results in the production of monoethylenic C_{20} and C_{22} unsaturated fatty acids as well as fatty alcohols in the head and blubber oils of the sperm whale.[307] In addition, isovaleric acid occurs in substantial amounts in the head and body oils of the delphinidae[308] (porpoises and dolphins). Thus marine mammals present a complicated pattern of fatty acid composition in which the dietary fats have been considerably modified. It is therefore difficult to separate the respective roles of the endogenous and exogenous fats in the formation of the depot fat.

C. Species that deposit endogenous fat only

A further differentiation in fat metabolism is exemplified in ruminant species. Here the organism largely loses the power to deposit fat from the diet. Carbohydrates, hemicellulose, cellulose and protein are broken down to volatile acids. The fate of the dietary fat is not precisely known. It may also be broken down by the rumen bacteria or in part hydrogenated as in the case of dietary linolenic acid. This leads to the formation of minor proportions of *trans* acids characteristic of ruminant species. It is evident that di- and polyethenoid C_{18}, C_{20} and C_{22} unsaturated acids when present in the diet are not deposited to any marked degree in the depot fat, and the dietary fat has no longer any appreciable influence on the composition of the depot fat. The result of the change in fat metabolism is that the depot fat now consists almost entirely of endogenous fat with palmitic, stearic and oleic acids as the major constituents. However, it has been shown that, if fat containing acids with odd numbers of carbon atoms is fed to ruminants, these acids are not destroyed but are deposited. A minor proportion of these acids is, however, dehydrogenated in the Δ^9-position.[243] This indicates that fatty acids which escape destruction or modification in the digestive tract of ruminants may be deposited in the depot fats as in non-ruminants. It also shows the ability of the organism to dehydrogenate fatty acids.

From the work of ANDERSON[309] it is apparent that bacilli synthesize lipids from non-fatty sources. The lipids are, however, non-glyceridic in nature, and contain for the most part, branched-chain fatty acids in contrast to the straight-chain even-numbered carbon atom acids characteristic of the rest of animal and plant life.

The changes in fat metabolism during the course of evolution illustrate the loss and gain in turn of the capacity to synthesize and deposit fat as the organism proceeds from a less specialized to a more specialized form.

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9

THE TRIGLYCERIDE COMPOSITION OF NATURAL FATS

R. J. Vander Wal

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9

THE TRIGLYCERIDE COMPOSITION OF NATURAL FATS

*R. J. Vander Wal**

One of the characteristics of the natural fats is the wide variation in physical properties to be found among members containing the same or similar fatty acids. Much of this variation is the result of differences in the proportions of the constituent acids, but these differences are not the only cause. For instance, the constituent fatty acids of cacao butter and mutton tallow each include about 60 per cent of saturated acids. Cacao butter contains about 35 per cent of C_{18} and 25 per cent of C_{16} saturated acids whereas mutton tallow contains approximately 30 per cent of C_{18}, 25 per cent of C_{16} and 5 per cent of C_{14} saturated acids.[1] In spite of the fact that mutton tallow contains less C_{18} and more lower melting C_{14}–C_{16} acids, its melting range is much higher than that of cacao butter. There is, however, over ten times as much GS_3† in mutton tallow as there is in cacao butter and the presence of such a large proportion of high-melting triglyceride accounts for the higher melting range of mutton tallow. In this instance the compositions of the component glycerides play a dominant role in the determination of melting ranges of the fats.

Another important characteristic of the natural fats is the remarkable constancy of physical properties shown by samples of the same kind of fat derived from various sources. Most samples of lard, for instance, melt (liquefy)‡ between 33°C and 46°C, have saponification values between 190 and 202, and iodine values between 53 and 77. The corresponding values for coconut oil are: melting point, 23°C to 26°C; saponification value, 250 to 264; and iodine value, 7·5 to 10·5. For menhaden oil the saponification value usually lies between 189 and 193 and the iodine value between 148 and 160.

The relative constancy of the physical properties of these natural fats indicates that the structure and constitution are constant, regardless of source, within certain fairly definite limits. It indicates also that the same regulating circumstances exist in each individual member of the species of plant or animal producing the fat, and that these vary to only a small extent between individuals.

* Armour and Company, Chicago, Illinois.

† The following abbreviations will be employed in the text:

G—Glyceryl radical.
S—Saturated acid(s) or saturated acid radical(s) according to context.
U—Unsaturated acid(s) or unsaturated acid radical(s) according to context.
A—Azelaic acid radical unless otherwise identified.

‡ Natural fats being mixtures do not, properly speaking, have a single definite melting point. The melting point is really the temperature at which the fat becomes completely liquid, by solution as well as by actual melting.

The Even Distribution Theory

What are the patterns of glyceride-type distribution in the various natural fats? How are individual fatty acids distributed among these types, and what regulating mechanisms determine the compositions of the natural fats? The answers to these questions are difficult to find, but because of the developing interest in adaptation and synthesis of fats for specific purposes they are important. This chapter is devoted to a discussion of modern theories of the glyceride structure of fats with particular emphasis on the most recent one, the "restricted random distribution" theory of KARTHA.

I. THE EVEN DISTRIBUTION THEORY

The first of the modern theories of the glyceride structure of fats was that which resulted from the extensive and enormously useful studies of HILDITCH and his co-workers. From their investigations was evolved the well-known theory of "even distribution" of fatty acids among the molecules of natural fats. According to this theory the component fatty acids in a natural fat tend to be distributed as far as possible among all the triglyceride molecules. The "phenomenon of even distribution" was recently stated by HILDITCH[2] as follows:

"1. When a given fatty acid A forms about 35 per cent (mole) or more of the total fatty acids (A + X) in a fat, it will occur at least once, $G(AX_2)$, in practically all the triglyceride molecules of the fat in question.

"2. If it forms from about 35 to about 65 per cent (mole) of the total fatty acids (A + X), it will occur twice, $G(A_2X)$, in any given triglyceride molecule in some instances, and of course more frequently the higher the proportion of this acid in the total fatty acids.

"3. If it forms 70 per cent or more of the total fatty acids, the remaining fatty acids (X) can at most only form mixed glycerides $G(A_2X)$, and the excess of A then, and broadly speaking then only, appears as a simple triglyceride, $G(A_3)$.

"4. A minor component acid which forms much less than about a third of the total fatty acids (e.g. 15 per cent or less), will not occur more than once in any triglyceride molecule (and, of course, not at all in many of the triglyceride molecules)."

Although the rule holds, at least approximately, for vegetable fats, it is not applicable to the structure of animal body fats rich in stearic acid such as those of sheep and oxen. Nevertheless, BANKS and HILDITCH[3] have advanced the opinion that even these fats may have been originally synthesized according to the rule of even distribution. According to this hypothesis the fats first synthesized are oleoglycerides which are preformed in accordance with the principle of even distribution. These more highly unsaturated fats are then partially hydrogenated to produce the characteristic, more saturated glycerides of the stearic-rich animal body fats.

Aside from all other considerations the HILDITCH theory of the glyceride structure of fats has a great weakness, in that it suggests no mechanism by which the fatty acids are distributed evenly among the triglyceride molecules.

II. The Random Distribution Theory

Longenecker[4] and Norris and Mattil[5] have expressed the opinion that animal fats may conform to a random distribution pattern, and Dean[4] has pointed to an indication that in the fats of the larger land animals the fatty acids are united indiscriminately with glycerol molecules. Banks and Hilditch[3] believe that even these fats may have been synthesized originally according to the even distribution pattern, as was stated earlier. There is experimental evidence that some fats, such as the stearic-rich animal fats, the milk fats and some vegetable fats do conform nearly to a random distribution pattern. Norris and Mattil suggest that there may be a basic difference between the synthetic action of plant and animal enzymes, whereby the fatty acids in animal fats are distributed essentially at random whereas those in vegetable fats are distributed otherwise.

If it be assumed that in animals the enzymes responsible for the synthesis of the triglycerides are capable of promoting either ester-interchange or a state of equilibrium between the fatty acids and glycerol on the one hand and the triglycerides and water on the other, random distribution of the fatty acids in the stearic-rich animal fats could be readily explained because no other state of distribution could exist under such circumstances. The two-step process suggested by Banks and Hilditch[3] whereby preformed oleoglycerides are converted to the final mixture by partial hydrogenation seems less likely to result in random distribution.

Norris and Mattil suggest no mechanism for the specific action they attribute to plant enzymes. They also do not explain why in some vegetable fats, such as nutmeg butter or dika fat, the fatty acids may be distributed approximately at random as in the animal and milk fats.

The random distribution pattern obviously cannot be applied to the structure of fats in general, and the supplementary hypothesis of a difference in action between plant and animal enzymes seems somewhat inconsistent. The reader is referred, for further discussion, to the excellent review of this and some other theories by Deuel.[6]

III. The Partial Random Distribution Theory

Doerschuk and Daubert[7] have recently presented good experimental evidence that the constituent triglycerides of corn oil conform to neither the even nor the random pattern of distribution. They find that their experimental values, which were determined by a crystallization procedure, conform more nearly to what they designate as a "partial random" pattern.

According to this scheme the pattern of even distribution is modified to include the occasional appearance in the same triglyceride molecule of two molecules of a fatty acid present in a concentration of less than 33 per cent of the total acids. According to the even distribution plan only one could be present under these circumstances in any triglyceride molecule. Furthermore,

contrary to the even distribution pattern, acids constituting 33 to 67 per cent of the total acids need not necessarily be present in every molecule of fat, according to the partial random theory.

According to the partial random pattern corn oil can contain 14 constitutionally different triglycerides, whereas only 6 are postulated by the even distribution theory and 20 by the random theory. DOERSCHUK and DAUBERT were able to separate their samples into 19 fractions by low-temperature fractionation. The glyceride structures of these fractions were calculated on the assumption that each contains only two adjacent glyceride types, such as GS_3–GS_2U or GS_2U–GSU_2. Combinations of the various calculations gave the glyceride structure of the fat.

The values calculated according to the partial random pattern agree well with those determined experimentally. Values calculated according to either the random or the even pattern do not agree so well. As DEUEL[5] has noted, of the 9 triglycerides postulated according to the partial random theory and not postulated by the random theory, 7 have been found in almost theoretical amounts by DOERSCHUK and DAUBERT.

Although the experimental evidence fits the proposed scheme of distribution very well in this case, acceptance of the partial random pattern of glyceride distribution as one of the major patterns must await its application to other fats. No mechanism accounting for the final mixture of glycerides has been postulated.

IV. THE RESTRICTED RANDOM DISTRIBUTION THEORY

The most recent theory of glyceride-type distribution is that proposed by KARTHA.[8], [9] Stated simply, KARTHA'S restricted random distribution theory is as follows:

1. GS_3 can be present in each kind of animal or plant fat to the extent only that it can remain fluid *in vivo*.

2. *In vivo* the fatty acids are distributed at random among the four glyceride types GS_3, GS_2U, GSU_2, and GU_3 provided the proportion of GS_3 thus produced can exist in the liquid state.

3. When the proportion of GS_3 which can exist in a liquid state *in vivo* is less than that which may be produced by unrestricted random distribution, the fatty acids are nevertheless distributed strictly according to chance among the glyceride types. The chance values will, however, vary according to the quantity of GS_3 present.

KARTHA has formulated a rule for calculating the glyceride-type distribution of any natural fat from the S and GS_3 contents. These values are percentages which can be determined in any particular sample of natural fat, and according to the theory all other proportions follow by the laws of chance.

He has determined the proportions of the four glyceride types in 27 natural fats by an improved method of analysis.[8], [9] Both experimental and calculated values are shown in Table I. The agreement between these values is remarkably close, in general.

V. Experimental Determination of the Glyceride-type Distribution of Natural Fats

Kartha has restricted his theoretical consideration and experimen . 1 to the four types of triglycerides, GS_3, GS_2U, GSU_2, and GU_3. No attempt has been made to subdivide these classes according to chain length, degree of unsaturation, or position of the substituents. His theories and calculations are based on the assumption that animal fats *in vivo* and vegetable fats during, at least, the ripening stage, are in a state of dynamic equilibrium with exchange of the fatty acids going on constantly, resulting, according to Kartha, in random distribution except under specific circumstances.

a. General procedure

The general procedure by which Kartha has determined the distribution of the four glyceride types is as follows:

1. The sample is oxidized to produce a mixture of azelaoglycerides (glycerides containing azelaic acid, the oxidation product of unsaturated acids, in the places previously occupied by the latter). Each azelaic group contains a free carboxyl group and can therefore form salts with metals such as magnesium. Any GS_2U, GSU_2, and GU_3 originally present then, is converted to GS_2A, GSA_2, and GA_3, respectively. Any GS_3 present will, of course, not be oxidized because it contains no unsaturated acid groups.

2. The GS_2A is precipitated quantitatively as the magnesium salt. The precipitate is contaminated with the magnesium salt of GSA_2 and contains all the GS_3 originally present in the sample. The weight of the GS_3, determined separately, is deducted from the weight of the precipitate, leaving only GS_2A and GSA_2. The percentages of each of these can be calculated from their total combined weights, the total weight of the S, and the mean molecular weight of the latter, all of which must be determined.

3. The fatty matter containing the remainder of the GSA_2 and all of the GA_3 is recovered by acidification and extraction from the soluble magnesium salts in the filtrate. All the S in this fraction is present as GSA_2, and the proportion of the latter can be calculated from the weight and mean molecular weight of the S. This portion of GSA_2 is added to the weight of that found in the insoluble fraction to give the total weight of GSA_2.

4. The proportions of the parent compounds GS_2U and GSU_2 can now be calculated from the known weights of GS_2A and GSA_2. It is assumed that the U is oleic acid unless the mean molecular weight of the U is known.

5. The proportions of GS_3, GS_2U, and GSU_2 are deducted from the original sample, leaving the proportion of GU_3 present therein.

b. The oxidation procedure

A key factor in the development of the Kartha theory of restricted random distribution was the early discovery[8], [9] that the azelaoglycerides are susceptible to hydrolysis by alkalies, thereby producing neutral compounds which separate with the GS_3.

In the method for the determination of GS_3 described by HILDITCH, the unsaturated molecules are oxidized to azelaoglycerides by means of powdered potassium permanganate and dry acetone. Some alkali is produced in the course of the reaction, and in later manipulations alkaline solutions are employed. Consequently, according to KARTHA, determinations of GS_3 by this method are likely to be erroneous because the neutral hydrolysis products of the azelaoglycerides separate with, and are determined as, GS_3.

The evidence presented by KARTHA[8], [9] for the inaccuracy of the Hilditch procedure consists of three experiments in which fats, the constitution of which was satisfactorily established, were oxidized by this method and the resultant products examined. In each case the results indicated that azelaoglycerides were actually decomposed by hydrolysis.

There is further evidence that the method used by HILDITCH for the estimation of GS_3 in fats is not entirely accurate. Only traces of GS_3 could be detected by crystallization procedures in cacao butter[10] and Borneo tallow,[11] whereas HILDITCH and colleagues reported 2·5 per cent of GS_3 in cacao butter[12] and 5 per cent in Borneo tallow.[13] In animal tallows, RIFMENSCHNEIDER *et al.*[14] and KARTHA[8], [9] found that GS_3 values by crystallization are appreciably below chance values, sometimes by as much as 5 to 8 per cent, whereas values reported by HILDITCH and colleagues[3], [15] are generally 2 to 5 per cent higher than chance values. KARTHA[16] found that it is possible to calculate the GS_3 contents of C_{16}–C_{18} fats directly from the saturated-acid content and the melting points of the fat and its mixed acids. In many cases the GS_3 content so determined is different from that determined by the method of HILDITCH, whereas it invariably agrees with values obtained by crystallization.

The remedy for the defect in the earlier oxidation procedure is simple.[9] It consists merely of adding and maintaining a small excess of acetic acid during oxidation* and avoiding drastic treatment with alkalis during the separation steps which follow.

The accuracy of the new procedure was tested in two experiments in which samples containing known quantities of GS_2U were oxidized. The amount of GS_2A which was found was almost identical with the theoretical quantity in each case.

KARTHA appears to have established with a reasonable degree of certainty

* Details of the oxidation procedure are as follows[8]:

Five grammes of the fat are dissolved in 200 cc of acetone, and 12 cc of glacial acetic acid (sufficient to combine with all of the potassium in 31 gm of $KMnO_4$) is added. The oxidation is carried out according to the Hilditch procedure except that after each 16 gm of $KMnO_4$ has been added, 6 cc of acetic acid is also added. Thus the concentration of acetic acid is always between about 3 and 6 per cent. When the reaction slows, it is continued at the boiling point on a water bath. When the permanganate colour disappears very slowly, as when 1 gm is not decolorized on boiling for 45 minutes, the oxidation is stopped. Usually by this time, 8 to 10 gm of $KMnO_4$ per gramme of fat will have been added. More would have been required for drying oils.

The acetone is removed by distillation, the last traces being eliminated with reduced pressure, by means of a water pump. About 150 cc of water is added and the mixture is heated on a water bath with agitation until the mass breaks up into a suspension. The manganese dioxide is then dissolved by addition of small quantities of sodium bisulphite and sulphuric acid alternately, as usual. The resultant clear layer of fatty matter is separated by extraction with ether. The extract is washed free from mineral acids and the ether is removed.

that earlier analyses in which the GS_3 content was determined by the Hilditch oxidation procedure are subject to error. It also appears that he has found a more accurate method for oxidation of unsaturated fats. He does not use this procedure, however, for the determination of GS_3 but for preparation of the mixture of azelaoglycerides to which he later applies his separation process. He determines the GS_3 by another method.

c. The determination of GS_3

The GS_3 content was determined separately, by the following procedure.[9]

The sample is dissolved in 3 cm^3 of dry acetone per gm of fat, and the solution is held at 25° to 26°C for three days. The precipitate is filtered and washed with a little acetone at the same temperature. It is then dried under reduced pressure and recrystallized from acetone under the same solvent-ratio, time, and temperature conditions. The vacuum-dried residue is weighed and the iodine and saponification values are determined. From these values the amount of unsaturated acid, as oleic acid, and also the mean molecular weights (M.M.W.) of the S are calculated. From all the data and under the assumptions that the precipitate contains all of the GS_3 in the sample, that it consists only of GS_3 and GS_2U, and that the S in both has the same M.M.W., the proportions of GS_3 and GS_2U are calculated.

d. Separation of the oxidation products and treatment of the fractions

The products of oxidation cannot be separated satisfactorily, according to KARTHA, by means of their differing solubility in sodium bicarbonate solution because of the solubility of GS_2A.[17] When a dilute solution of bicarbonate is used to minimize this solubility the tendency for nonanoic acid to remain behind increases.

KARTHA found that the reported insolubility of the magnesium salts in water[18] is only partially correct. Magnesium triazelain is soluble to not less than 3 to 4 per cent in water at 30°C; the magnesium salts of GSA_2 show an appreciable though more sparing solubility, whereas the magnesium salts of GS_2A containing the higher acids are insoluble in water at 30°C.

Based on these findings the following method for separation of the oxidation products has been adopted.[9]

The oxidation product from 5 gm of fat is suspended in about 200 cm^3 of water (30°C) and a few drops of phenolphthalein are added followed by 5 per cent sodium carbonate solution until the solution is just alkaline. The volume is made up to 500 cm^3 with water (100 cm^3 per gm of fat oxidized), and 30 cm^3 of 10 per cent ammonium chloride solution is added and the solution mixed well. Finally 15 per cent magnesium sulphate solution is added until no more precipitate forms. The precipitate is allowed to settle for 5 to 10 minutes, is filtered on fluted filter paper, and is washed four times with 30 to 40 cm^3 portions of water. It is then transferred quantitatively to a flask and heated on a water bath with dilute sulphuric acid until all the magnesium salts are decomposed and a clear oily layer separates. The mixture is then cooled and extracted

with ether, and the ether solution is washed free of mineral acids. The ether is removed and the residue is heated to constant weight under reduced pressure.

The product is hydrolyzed with alcoholic KOH, and the isolated acids are subjected twice to magnesium salt precipitation as in the Bertram method for determining saturated acids. The weights, iodine values, saponification values, and melting points of the separated acids are determined.

The combined filtrates are acidified and the acidic fractions are extracted with ether, which is removed by evaporation. The residue is hydrolyzed with alcoholic KOH and the higher saturated fatty acids are separated by BERTRAM'S method. Their weights, melting points, and saponification values are determined.

From the accumulated data and the GS_3 content as determined separately, the glyceride-type composition is calculated. The method of calculation will be given in the next section.

The method of separation is applicable only to fats containing mainly fatty acids of 16 or more carbon atoms, to which the Bertram separation can be applied.[19]

e. Detailed method of calculation of glyceride-type distribution

The method of calculation essentially as described by KARTHA[9] is as follows:

The fatty matter recovered from the precipitated magnesium salts will contain all the GS_3 originally present in the fat, any unoxidized GS_2U,* all the GS_2A, and usually a variable amount of GSA_2. The weight of the GS_3 present in the fat sample, found independently, and that of the unoxidized GS_2U are deducted from the total. The balance consists of GS_2A and GSA_2.

The weight of unoxidized GS_2U (W_2) is calculated from the mean molecular weight (M) of the S found in the precipitated magnesium salts, and the weight of the unsaturated acids (W_1), assumed to be oleic, in the same fraction.

$$W_2 = W_1 \frac{(2M + 282 + 38)}{282}$$

The values of M and W_1 and the weight of the S in the precipitated magnesium salts are calculated from the weight, saponification value, and iodine value of the acids separated from the latter, as described in the previous section.

The combined weights of the GS_2A and GSA_2 and the weight and mean molecular weight of the S in the fatty matter recovered from the precipitated magnesium salts are now known. The weight of the S in the whole mixture minus the weights of the S present as GS_3 and unoxidized GS_2U gives the weight of S present in the mixture of GS_2A and GSA_2. From these data the percentages of GS_2A and GSA_2 can be calculated. It is assumed that the S in both has the same M.M.W., which has been found to be true in a large number of fats.[8], [17]

* This fraction should contain any GSUA present in the oxidation product. It is assumed, however, that none is present. The close agreement between experimental and calculated results (Table 1) indicates that the assumption is correct.

From the weights of GS_2A and GSA_2 found in the precipitated magnesium salts, the weights of the GS_2U and GSU_2 from which they were derived may be calculated. It is assumed that the U is oleic acid. If the M.M.W. of the U in the fat is known, this value may be substituted. The weight of GS_2U thus obtained is added to the weight of the unoxidized GS_2U to give the total weight of the GS_2U in the original fat sample.

The filtrates from the precipitated magnesium salts contain part of the GSA_2, the remainder having been precipitated with the insoluble fraction. The weight of the S in the recovered fatty material from the filtrates is determined by isolation of the fatty acids by the procedure described in the previous section. It is present in the filtrate entirely as GSA_2 and from its weight the weight of the GSA_2 can be calculated. The weight of GSU_2 from which the GSA_2 was derived can be calculated from the result. The total GSU_2 in the fat is the sum of that found in the soluble and insoluble fractions of the magnesium salts. The per cent of GU_3 is found by difference.

The total S in the fat is the sum of that found in the GS_3, GS_2U, and GSU_2 fractions. It can be determined also by Bertram separation of the acids produced by hydrolysis of the product of acetic acid-permanganate-acetone oxidation. It is also the sum of that found in the fatty matter recovered from the soluble and insoluble magnesium-salt fractions.

The amount of fat required for glyceride-type analysis by this method is small. The time required is also relatively short. Duplicate determinations rarely show more than 0·5 per cent difference in the GS_2U content.

The results of application of the method to the glyceride-type estimation of 27 fats of saturated acid content varying from 8 to 99 moles per cent are shown in Table 1.

Kartha's *rule for calculation of the glyceride-type distribution of natural fats from the S and GS_3 content*

Table 1 shows the results of the analysis of 27 natural fats by Kartha's oxidation and separation methods. It also shows the results of calculation of the glyceride-type distribution from the S and GS_3 content. These calculations were made in accord with a rule which will be stated later in this section.

In Table 2 are presented the apparent glyceride-type compositions of 46 natural fats, calculated by Kartha from crystallization data, according to the procedure of Hilditch and Meara.[21] The data are from various sources.[14], [22]–[53] In many cases the mole per cent of saturated acids (Sm) reportedly found by analysis does not agree with the glyceride-type composition found by calculation from fractional crystallization results; in such cases the mole per cent of S has been calculated from the results of glyceride-type analysis by the latter method.

In Table 2 are given also the results obtained by treatment of the same data according to the new rule for calculation of glyceride-type distribution, which will be presented later in this section. These results were calculated from the reported GS_3 contents and the reported or calculated S contents.

*Table 1. Glyceride-type composition of some natural fats as obtained by Kartha's method**

Fat	*S* (mole %)	*Found: present procedure (mole %)*				*Calculated: present procedure (mole %)*			*Calculated: even distribution (mole %)*			
		GS_3	GS_2U	GSU_2	GU_3	GS_2U	GSU_2	GU_3	GS_3	GS_2U	GSU_2	GU_3
Apricot kernel oil	8	0	1	22	77	1	22	77	0	0	24	76
Linseed oil	13	0	5	29	66	5	29	66	0	0	39	61
Sesame oil	16	0	6	36	58	7	34	59	0	0	48	52
Samadera indica oil	16	0	6	37	57	7	34	59	0	0	48	52
Peanut oil	20	0	9	42	49	10	40	50	0	0	60	40
Foenugreek seed oil	20	0	11	38	51	10	40	50	0	0	60	40
Almond kernel oil	22	0	11	47	42	13	42	45	0	0	66	34
Corn oil	22	0	12	43	45	13	42	45	0	0	66	34
Cottonseed oil	23	0	13	44	43	14	41	45	0	0	69	31
Calophyllum oil	23	0	15	39	46	14	41	45	0	0	69	31
Shark liver oil	24	0	14	43	43	15	42	43	0	0	72	28
Pongamia glabra oil	26	0	15	48	37	18	44	39	0	0	78	22
Duck fat	31	2	19	48	31	21	44	33	0	0	93	7
Thevetia nerufolium oil	33	0	26	47	27	27	45	28	0	0	99	1
Terminalia cattappa oil	38	trace	34	46	20	35	44	21	0	14	86	0
Ter. bellerica oil	40	trace	39	41	20	39	43	19	0	19	81	0
Neem oil	40	trace	39	41	20	39	43	19	0	19	81	0
Mowhra oil	43	trace	47	36	17	45	39	16	0	29	71	0
Sarcostigma kleinii fat	50	trace	59	30	11	58	33	9	0	59	50	0
Palm oil	54	9	54	27	9	53	30	8	0	62	38	0
Vateria indica fat	57	1	76	18	5	73	23	3	0	71	29	0
Garcinia cambogia fat	58	1	78	18	3	78	18	2	0	74	26	0
Garcinia indica fat	61	1	84	13	2	82	15	1	0	83	17	0
Ox depot, Australian	61	16	55	26	3	56	24	4	0	83	17	0
Ox depot, Indian	63	17	58	22	3	59	21	3	0	89	11	0
Goat depot, Indian	69	22	63	14	1	64	13	1	10	90	0	0
Actinodaphne hookeri fat	99	97	3	0	0	3	0	0	97	3	0	0

* Adapted from KARTHA.[111]

*Table 2. Glyceride-type composition of natural fats by fractional crystallization methods**

Fat	S (mole %)	Found by fractional crystallization (mole %)				Calculated present hypothesis (mole %)		
		GS_3	GS_2U	GSU_2	GU_3	GS_2U	GSU_2	GU_3
Almond oil[22]	6	—	—	17	83	1	15	84
Safflower seed oil[23]	6	—	—	18	82	1	15	84
Niger seed oil[24]	9	—	—	26	74	2	22	76
Olive oil I[22]	15	—	—	45	55	6	32	62
Tobacco seed oil[23]	15	—	—	45	55	6	32	62
Gray seal oil[25]	17	—	—	51	49	8	35	57
Peanut oil[22]	19	—	1	56	43	10	38	52
Olive oil II[22]	21	—	—	61	39	12	39	49
Neats foot oil[26]	22	—	7	51	42	13	40	47
Herring oil[27]	23	—	4	61	35	14	41	45
Cottonseed oil I[28]	28	—	13	59	28	21	43	36
Sacred baboon fat[29]	29	—	6	74	20	22	34	44
Cottonseed oil II[30]	31	—	19	56	25	25	44	31
Whale oil[31]	31	—	9	75	16	25	44	31
Neem oil[32]	32	—	14	67	19	26	44	30
Sapota oil[33]	34	—	8	87	5	28	45	27
Ceylon bear fat[13]	34	2	7	82	9	25	44	29
Lard[14]	37	2	26	54	18	31	44	23
Minusops hekelii fat[34]	42	1	34	55	10	42	41	16
Mowhra oil[35]	43	1	28	71	0	43	40	16
Palm oil, Grand Bassa[36]	45	4	41	43	12	42	39	15
Pig external[37]	46	5	32	60	3	42	39	14
Shea butter[38]	46	4	36	55	5	43	39	14
Lophira alata fat[39]	46	1	37	62	0	49	37	13
Hodgsonia capniocarpa fat[40]	50	2	60	25	13	55	34	9
Garcinia morella fat[41]	50	2	46	51	1	55	34	9
Kangaroo fat[29]	51	1	51	48	0	59	32	8
Ewe external[42]	51	5	42	53	0	52	34	9
Palm oil, Belgian Congo[43]	51	5	44	51	0	52	34	9
Mangifera indica[44]	51	14	24	61	1	38	36	12
Pig perinephric[37]	52	9	43	45	3	48	43	10
Palm oil, Cameroons[36]	55	8	54	32	6	55	31	9
Shorea robusta fat[45]	55	2	63	34	1	66	27	5
Allanblackia stuhlmannii[46]	56	1	66	33	0	70	25	4
Allanblackia parviflora[47]	57	2	66	32	0	70	25	3
Vateria indica fat[48]	58	2	69	29	0	72	24	2
Ewe perinephric[42]	58	14	48	38	0	52	28	6
Beef tallow[14]	58	15	46	37	2	51	28	6
Garcinia indica fat[41]	59	1	76	21	2	77	20	2
Heifer, English[49]	61	17	49	34	0	54	25	5
Allanblackia floribunda[47]	62	2	81	17	0	83	15	1
Cacao butter[50]	61	2	77	21	0	80	17	1
Phulwara butter[51]	62	8	69	23	0	72	18	2
Borneo tallow[52]	63	5	79	16	0	79	16	1
Ox depot fat, Calicut[53]	69	28	52	20	0	53	17	2
Ox depot, Bombay[53]	75	36	52	12	0	54	9	1

* Adapted from KARTHA[20]. Numbers in parentheses are references.

KARTHA's restricted random distribution theory has been stated earlier in this chapter. Because it is based on the laws of probability and measurable quantities it is susceptible to mathematical treatment. KARTHA has stated a rule[8], [20] based on the above-mentioned theory, by which the glyceride-type distribution of any natural fat can be calculated from the percentages of S and GS_3 present in the fat. The rule follows:

> The glyceride-type composition of any natural fat is that obtained by interchange according to chance of one-third of the saturated acids represented by the difference between the GS_3 content required by chance and that actually present with the unsaturated acids in the fat, without allowing the formation of any further GS_3.

The method of calculation as stated by KARTHA[20] follows:

Let the amount, in units per cent, of S substituting for U in GSU_2 be a, and that substituting for U in GU_3 be b. Then the amount of GSU_2, converted to GS_2U, is equal to $3a$. Similarly $3b$ moles of GU_3 may be assumed to be converted to GSU_2. The substitution of U by S in GU_3 will give rise to a small amount of GS_2U also, but this may be disregarded for practical purposes. The factor (GS_3 chance $-$ GS_3 actual) is, in effect, entirely converted to GS_2U by this interchange. Hence the final composition of the fat will be as follows:

$$GS_2U \text{ actual} = GS_2U \text{ chance} + (GS_3 \text{ chance} - GS_3 \text{ actual}) + 3a$$

$$GSU_2 \text{ actual} = GSU_2 \text{ chance} - 3a + 3b$$

$$GU_3 \text{ actual} = GU_3 \text{ chance} - 3b$$

GS_3 actual has been found by experiment

KARTHA illustrates the application of the method of calculation by the following example.[20]

The acid components of a sample of low-melting vegetable fat containing zero mole per cent of GS_3 have been shown to contain 50 mole per cent of saturated acids. What is the mole per cent of each of the four glyceride types GS_3, GS_2U, GSU_2, and GU_3 in the mixture, according to the rule stated previously?

If the S were distributed at random, the glyceride-type content would be 12·5 per cent GS_3, 37·5 per cent GS_2U, 37·5 per cent GSU_2, and 12·5 per cent GU_3. Actually no GS_3 has been found, so the difference between GS_3 chance and GS_3 actual is 12·5 per cent.

According to the rule, one-third of the saturated acids in this quantity of GS_3 will be interchanged according to chance with unsaturated acids in the fat without formation of more GS_3. In other words, 12·5/3 = 4·2 mole per cent of S from GS_3 is interchanged, according to chance, with an equivalent quantity of U present in the chance values of GSU_2 (37·5 per cent) and GU_3 (12·5 per cent). The U in GS_2U will not be replaced since this would result in formation of more GS_3, which is contrary to the rule.

Two-thirds of the component fatty acids in GSU_2 are unsaturated, so the unsaturated acids represented by the chance value of GSU_2 (37·5 per cent) comprise (37·5)(2)/3 = 25 per cent of the total fatty acids in the sample. Similarly the unsaturated acids represented by the chance value of GU_3 (12·5 per cent) comprise 12·5 per cent of the total fatty acids in the fat. The ratio of the unsaturated fatty acids in GSU_2 chance to those in GU_3 chance is therefore 25 : 12·5 or 2 : 1, and the 4·2 per cent of S will be distributed in that ratio. Therefore, 2·8 of the 4·2 per cent of S will replace one U in GSU_2 and 1·4 per cent will replace one U in GU_3. These are values a and b in the formulae given earlier. Theoretically (1·4)(100)/12·5 or about 12 per cent of U present in GU_3 chance is interchanged with S. Therefore (12)(12)/(100)(100), or about 1·5 per cent of the total GU_3 molecules are converted to GS_2U instead of to GSU_2. This quantity represents only (1·5)(12·5)/(100)(100), or 0·17 per cent of the total quantity of fat and can be ignored.

The glyceride composition is finally calculated by substitution in the formulae as follows:

$$GS_3 = \text{zero (by analysis)}$$
$$GS_2U = 37{\cdot}5 + 12{\cdot}5 + (3)(2{\cdot}8) = 58{\cdot}4 \text{ mole per cent}$$
$$GSU_2 = 37{\cdot}5 - (3)(2{\cdot}8) + (3)(1{\cdot}4) = 33{\cdot}3 \text{ mole per cent}$$
$$GU_3 = 12{\cdot}5 - (3)(1{\cdot}4) = 8{\cdot}3 \text{ mole per cent}$$

The results of many such calculations are shown in Tables 1 and 2. It is evident from Table 1 that there is a high degree of correlation between KARTHA's experimental results and those calculated from the S and GS_3 content according to KARTHA's rule. Assuming that the experimental results are correct, the startlingly close correlation between experimental and calculated values constitutes strong evidence of the correctness of KARTHA's theory. Both calculated and experimental values differ markedly, in most cases, from the values calculated according to the rule of even distribution. There is general disagreement between the results calculated from fractional-crystallization data and those calculated from the given S and GS_3 contents according to KARTHA's rule.

The following characteristics of the data in Tables 1 and 2 have been noted by KARTHA.[20] Slight changes have been made in the text:

1. Throughout almost the entire series, values for GS_2U and GU_3 are higher than those calculated from crystallization data or according to the rule of even distribution, whereas those for GSU_2 are lower. In cases where the amount of GU_3 present is small, results agree well with those derived from crystallization data.

2. Whereas crystallization data do not show the presence of any GS_2U in oils with a saturated-acid content of less than 22 mole per cent, KARTHA's results show that GS_2U is present, and with an S content between 12 and 22 mole per cent the proportions of GS_2U are considerable.

3. The KARTHA results show that in oils with an S content less than about 30 mole per cent, where GS_3 according to chance would be less than 2 to 3

per cent, the proportions of all the glyceride types are approximately those required by chance, regardless of biologic source or fatty-acid composition.

4. In oils and fats with very small GS_3 contents, the results obtained by KARTHA show a progressive increase in the GS_2U content with increase in saturated acid content until the latter reaches about 66·6 per cent. No such regular increase in GS_2U content is shown by the results derived from crystallization data, which frequently give widely differing GS_2U values for fats of the same GS_3 and S content.

5. Until the unsaturated-acid content falls below about 45 mole per cent, the GU_3 values found by the KARTHA procedure agree closely with chance values. Where the unsaturated-acid content falls below this level, the GU_3 values tend to be lower than chance values by an amount dependent on the difference between GS_3 chance and GS_3 actual. No such results are found in the calculations from crystallization data. These data frequently show widely differing GU_3 contents for oils and fats with the same unsaturated acid content.

6. According to the rule of even distribution the GSU_2 content of a fat can be 100 per cent. Crystallization data have indicated values as high as 87 per cent, although generally they indicate values above about 55 mole per cent for fats containing 19 to 48 mole per cent of saturated acids (Table 2). The results obtained by KARTHA (Table 1) show no values for GSU_2 content higher than 48 per cent. Further, as with GU_3, the GSU_2 values agree closely with chance requirements until the unsaturated acid content falls below about 45 mole per cent. Below this, they tend to be lower than chance values by an amount dependent on the difference between GS_3 chance and GS_3 actual.

7. KARTHA's results show that when GS_3 actual is about the same as GS_3 chance, the values for all the other glyceride types also agree well with those required by chance. No reliable case has been found where GS_3 actual is higher than GS_3 chance. When GS_3 actual is lower than GS_3 chance the values for all other types also differ from chance values. GS_2U, GSU_2, and GU_3 values always differ in the same way from chance values when GS_3 actual is less than GS_3 chance. There is always an increase in the GS_2U content to above chance values and decrease in the GSU_2 and GU_3 values to below chance values. These relationships are not evident among the results derived from crystallization data.

The observations are in agreement with the restricted random theory and with KARTHA's rule for calculation of glyceride-type distribution. They constitute further evidence for the correctness of the theory.

VI. Factors Governing the Content of Fully Saturated Triglycerides

If the fatty acids in natural fats are actually distributed at random except for the restriction which may be imposed through limitation of the GS_3 content,

why is it that in some cases the restriction occurs while in other cases it does not? A mechanism must exist whereby the GS_3 in some fats is limited to less than chance proportions, whereas in all others, according to KARTHA, it is limited only to the quantity which can be produced by completely random distribution of all the fatty acids.

KARTHA has advanced a simple explanation which is in accord with several basic facts and is supported by a considerable amount of evidence taken from existing literature.

According to KARTHA's hypothesis the reason why GS_3 is restricted to less than chance values in some natural fats is that it (and all other glyceride types) can be present *in vivo* only to the extent that it can remain in a liquid state and that in the fats in question the limit of fluidity is below the chance value. No more GS_3 can, therefore, be present since any increment would be solid. Any excess S in the fat must then be distributed among the remaining glyceride types, which being more unsaturated and hence lower melting and more soluble can exist in larger proportions in the liquid state.

When the quantity of GS_3 which can be formed by complete random distribution can remain liquid *in vivo*, this and all other glyceride types will be present in chance proportions. If, on the other hand, the circumstances *in vivo* are such that less GS_3 can exist as a fluid in the fat, then the fatty acids will still be distributed according to chance, but the chance values will vary according to the quantity of GS_3 present.

If KARTHA's hypothesis is correct and glyceride-type distribution is dependent on the extent to which GS_3 can remain in a fluid state in the fatty substrate then the proportions of GS_3 in fats should increase as its nature and that of the substrate increasingly favour its existence as a fluid. To put it another way, the greater the tendency of the GS_3 to become fluid the more of it should be found in a natural fat.

The extent to which GS_3 can remain in a fluid state in natural fat *in vivo* is dependent, according to KARTHA, on at least two factors. These are (1) the chain length of the GS_3 and (2) the melting point of the GS_3.

It was shown by KARTHA[54] that the "melting point" (maximum limit of the melting range) of a mixture of a particular GS_3 in a particular non-GS_3 triglyceride substrate is a function of the GS_3 content. The greater the proportion of GS_3 the higher is the melting point. This demonstrates that solid GS_3 becomes liquid by solution in the substrate and not by melting. The solubility of the GS_3, according to KARTHA, depends partly on its melting point.

KARTHA states that the "melting point constant," defined as the difference in melting point of a fat produced by decreasing the GS_3 content by one half, increases with decreasing chain length of the GS_3. This shows, according to KARTHA, that decrease in chain length (resulting also in a decreased melting point) increases the solubility of the GS_3 in the substrate, beyond that resulting from the decreased melting point. On the basis of this evidence KARTHA concludes that both melting point and chain length of the GS_3 are factors governing the extent to which it can remain liquid in fats *in vivo*.

The melting points of whole fats *in vitro* are not a reliable criterion by which to estimate the extent to which GS_3 can remain fluid in them *in vivo*. The data in Table 3 show a rather poor correlation between the GS_3 content of a fat and the melting point of the whole fat *in vitro*.

Animal tallows, according to KARTHA, are always present in the liquid state only in living adipose tissue,[55] the normal temperature of which is frequently 10 to 15°C below the melting point of the refined fat. If so, it is evident that for some reason fats can exist in the fluid state *in vivo* at temperatures much below the normal melting point. This circumstance obviously has a bearing on the amount of GS_3 which can remain fluid *in vivo*.

If it be conceded that fats are present only as liquids *in vivo* there is little correlation between the melting points of the refined whole fats and the extent to which GS_3 can remain fluid in them *in vivo*. There is, however, good correlation between the chain length (and melting point) of the GS_3 and the extent to which it can remain fluid *in vivo*. This relationship is shown in Table 3.[56] The data also show that in a group of fats of approximately the same melting point, the GS_3 content increases with decrease in chain length. Decrease in chain length increases the solubility and decreases the melting point of the GS_3 or, in other words, increases its tendency to exist as a fluid.

The data are taken from various sources,[1], [57] and show more or less accurately the composition of the saturated constituents of several natural fats. Some of the data are probably not strictly accurate and the melting ranges were sometimes determined on different samples. Nevertheless the error is relatively small and the assembled data may be used for the purposes of these demonstrations.

Inspection of Table 3 will show that as the proportion of GS_3 increases from 1·4 to over 85 mole per cent of the total fat there is a corresponding decrease in the proportions of C_{18} and higher acids from 96 to 0 per cent. This is accompanied by an increase in the proportion of C_{14} and lower acids from 0 to 100 per cent. At the same time the proportion of C_{16} acids first increases and then decreases as the foot of the table is approached.

It is obvious that as the mole per cent of GS_3 increases, the proportions of higher-melting, more-insoluble C_{18} or higher acids in the total saturated acids decrease, to be replaced by lower-melting, more soluble, C_{16} acids. These in turn are replaced by still lower melting, still more soluble, C_{14} and lower acids.

The comparison between the first member of the series, rambutan tallow, and the next to the last member, dika fat, is particularly striking. Rambutan tallow, a tropical vegetable fat, contains only 1·4 per cent GS_3 and its saturated fatty acids are almost exclusively stearic or higher. Its melting range is 40 to 46°C. Dika fat, also a tropical vegetable fat, contains 79 per cent GS_3. The saturated fatty acids consist exclusively of C_{14} or lower members. The melting range, 41 to 42°C, is almost the same as that of rambutan tallow. The GS_3 actually found in rambutan tallow is only about 10 per cent of that which could be produced by completely random distribution, whereas dika fat contains all the GS_3 which could be produced under the same circumstance.

Interpreted according to the KARTHA theory, these data mean that in rambutan tallow the GS_3 content is greatly restricted because a higher proportion of this particular high-melting GS_3 could not remain dissolved in the substrate. Dika fat, on the other hand, contains more GS_3 because the latter is more soluble in the substrate owing to the shorter chain length of the component acids. It should be emphasized that the two fats have similar melting ranges and are both tropical vegetable fats.

The same relationships hold throughout the series of fats shown in the table. As the proportion of lower-melting, more soluble acids in the saturated portion increases there is a corresponding increase in the GS_3 content as determined experimentally. The difference between the GS_3 required by random distribution and that actually found decreases as the proportion of GS_3 becomes larger. This means, according to KARTHA's hypothesis, that as the chain length of the GS_3 decreases, thereby decreasing its melting point and increasing its solubility, there is less restriction on its formation in the fat.

The increase in GS_3 content of the fats shown in the table is not due to increased proportions of saturated fatty acids because, with the exclusion of chicken fat and the last six members of the series, the maximum variation in saturated acid content is 14 per cent (49 to 63 per cent). All other relationships exist in this curtailed series that exist in the whole series. The melting ranges are even more nearly the same.

It appears on the basis of this demonstration that KARTHA's theory, that the formation of GS_3 *in vivo* is restricted to the proportion which can remain in a liquid state *in vivo*, has experimental support.

A part of KARTHA's theories of glyceride composition and synthesis is the assumption that natural fats *in vivo* are in states of dynamic equilibrium during the period in which their structure is defined, and this assumption is implicit in his rule for calculation of glyceride-type distribution. This assumption is in agreement with the evidence of several others[58] which indicates that lipolytic esterification by enzymes is reversible and proceeds according to the law of mass action. Lipolytic enzymes should then behave, in this respect, like ordinary esterification catalysts, promoting a reversible equilibrium, even though sometimes a restricted one, between reactants and reaction products. It is also possible that they may act as ester-interchange catalysts. Either mode of action can result in random distribution of the fatty acids, subject only to restriction such as that suggested by KARTHA.

KARTHA's assumption of dynamic equilibrium is in harmony with his observation[59] that fats of varying composition from ingested foods are deposited in adipose tissues of oxen and the milk fats of cows and buffaloes without changing the ratio of the GS_3 found to that which would be expected by chance distribution, indicating that the basic pattern of glyceride composition is unchanged.

The theories and assumptions agree with what appears to be the fact that fatty-acid synthesis and esterification take place in all vegetable and animal fat depots,[60] thus affording an explanation for the simultaneous appearance of fats of differing composition in various parts of the same organism.

*Table 3. The saturated components of some natural fats (approx.)**

Fat	Type	Component S fatty acids (approx.)			% S (mole)	% GS_2 actual (mole)	M.P. fat	% GS_2 chance
		% C_{16} or higher	% C_{14}	% C_{14} or lower				
Rambutan tallow	Trop. veg.	96	4	—	49·0	1·4	40–46°C	11·76
Pulasan fat	Trop. veg.	95	5	—	55·3	1·5	40–42	16·91
Garcinia indica fat	Trop. veg.	95	5	—	59	1·5	40–43	20·5
Allanblackia stuhlmannii fat	Trop. veg.	94	6	—	56	1·5	43–46	17·6
Chicken fat	Bird fat	20	79·71	0·29	34·0	2·5	32–34	3·93
Garcinia morella fat	Trop. veg.	85	14	1	50	2·7	34–37	12·5
Borneo tallow	Trop. veg.	63	35	2	63	4·5	34–39	25·0
Phulwara butter	Trop. veg.	5·4	92	2·6	62	8·0	39–51	23·8
Palm oil (Belgian Congo, African)	Trop. veg.	11	86	3	51	10·3	27–50	13·3
Hog fat (perinephric)	Swine fat	37·31	58·42	4·26	46·9	13·2	33–46	10·3
Beef tallow (N. American)	Bovine fat	26·8	58·2	15·0	50·0	13·9	40–48	12·5
Camel milk fat	Ruminant milk fat	14·6	50·0	35·4	62·6	25·6	35·3SP	24·5
Mutton tallow	Sheep fat	48·03	42·95	9·02	61·0	26·6	44–51°C	22·7
Cow milk fat (English, spring pasture, 1929)	Ruminant milk fat	14·9	39·5	45·4	61·9	27·2	28–35	23·7
Stillingia tallow (U.S., Florida)	Trop. veg.	9·8	83·0	7·2	72·5	28·4	48·2SP	38·1
Babassu fat	Trop. veg.	1	9	90	82·5	63wt	24–26°C	56·1
Palm kernel fat	Trop. veg.	4	6	90	85·3	66	24–26	62·1
Nutmeg butter fat	Trop. veg.	—	6·7	93·3	90·2	73	42–45	73·4
Dika fat	Trop. veg.	—	—	100	91·7	81	41–42	77·1
Coconut fat	Trop. veg.	2	11	87	93·9	86	23–26	82·8

* From KARTHA.[166]

VII. The Mechanism by which Formation of Solid GS_3 is Prevented

The action of lipolytic enzymes in fats has been compared previously with that of ordinary esterification and ester-interchange catalysts. But if they act exactly like ordinary esterification catalysts they should produce solid GS_3 when the limit of fluidity is exceeded at any particular temperature. In theory, the ester interchange process can continue under some circumstances until almost all the fatty acids have been separated into solid GS_3 and liquid GU_3. If the enzymes do promote a state of equilibrium there must be some difference between their mode of action and that of ordinary esterification and ester-interchange catalysts which prevents them from ever producing solid GS_3.

Kartha[56] has advanced a tentative explanation based on five assumptions, which are:

1. The enzymes are colloidal.
2. They effect esterification of fatty acids and glycerol by forming stable intermediate complexes with the reactants which rearrange to form stable complexes with the reaction products. These complexes then break down to produce the reaction products and free the enzyme. Ester interchange is effected in a similar manner.
3. This process is reversible.
4. Neither complex can form unless the factors can form a continuous adsorbed film on the surface of the colloidal enzyme.
5. A solid factor cannot form a continuous adsorbed film.

If one of the reactants, such as GS_3, is a solid the continuous adsorbed film will not form and the complex also will not form. As a result no reaction will occur.

It has been shown by Willstätter and Waldschmidt-Leitz[61] that ricinus or pancreatic lipase has no effect on tristearin crystals, possibly because the solid triglyceride cannot form a continuous adsorbed film on the enzyme surface.

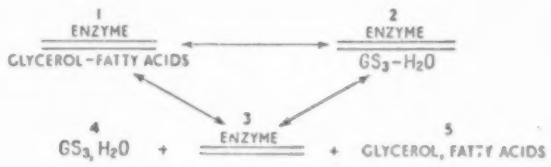
Collins[62] has demonstrated that lipolytic bacteria do not hydrolyze solid tristearin. Tofte[63] has shown that the rate of hydrolysis of hydrogenated fats at fixed temperatures by means of ricinus or pancreatic lipase decreases with increase in the melting point of the fat or, in other words, when more solid triglyceride is present. For fats of the same melting point, rates of hydrolysis increase as the temperature increases, presumably because of the resultant decrease in the proportion of solid fat.

There appears therefore to be some evidence in support of the theory that enzymes cannot promote reactions with solid fat because the latter cannot form a continuous adsorbed film on the surface of the enzyme. If the glyceride-water-enzyme complex cannot form, it obviously cannot rearrange to produce a fatty acid-glycerol-enzyme complex and no hydrolysis will occur. The same principle applies to ester interchange.

This is, of course, the reverse of the problem with which we are concerned. Instead of why solid glycerides are not hydrolyzed by enzymes, our concern is with the reasons enzymes cannot synthesize or otherwise produce solid triglycerides, assuming this to be true.

KARTHA suggests that the converse of the reason for the failure of enzymes to hydrolyze solid triglycerides may be the reason for the failure of enzymes to produce solid triglycerides. It may be, according to KARTHA, that the stable glycerol-fatty acids-enzyme complex rearranges to form the enzyme-GS_3-water complex only when the latter can form a continuous adsorbed film on the surface of the enzyme. He suggests that this can be done only if the GS_3 is in the liquid state. As a result GS_3 is produced only to the extent that the GS_3 in the complex can exist in the liquid state. GS_3, which is liquid under these circumstances, may also be liquid when freed from the complex. When the liquid film fails to form, no more GS_3 will be produced.

The hypothesis may be demonstrated by the following diagram in which the double lines represent enzyme surfaces:



There is evidence[61], [62], [63] that the counter-clockwise cycle

$$(4) + (3) \rightarrow (2) \rightarrow (1) \rightarrow (3) + (5)$$

does not operate unless both GS_3–H_2O and glycerol-fatty acid films can form on the enzyme surface.

KARTHA suggests that the clockwise cycle $(5) + (3) \rightarrow (1) \rightarrow (2) \rightarrow (3) + (4)$ also cannot operate unless the same situation exists and both GS_3–H_2O and glycerol-fatty acid films can form. He suggests further that these films can form only if the adsorbed materials are liquids. If the GS_3 is solid it cannot form a film and the cycle will not be carried out. As a result no solid GS_3 will be produced. He advances no experimental evidence for this hypothesis.

The theory of glyceride-type distribution advanced by KARTHA is supported by what appears to be good experimental evidence, but perhaps one of the strongest arguments in its favour is its remarkable completeness. Not only can he determine the glyceride-type distribution analytically with what appears to be good accuracy, but he can predict the distribution by calculation from simpler data. He supports his theory of glyceride-type distribution by a supplementary hypothesis, based on experimental evidence, explaining the mechanism by which the glyceride types are so distributed. Whether his conclusions are correct will be demonstrated eventually, but whatever the outcome the studies must be recorded as the most complete and ingenious yet produced.

Recently QUIMBY, WILLE, and LUTTON[64] have presented the results of

x-ray and thermal studies on lard, mutton tallow, and beef tallow which indicate that these fats conform to neither the random nor the restricted random theories.

LUTTON[65] and LUTTON and JACKSON[66] have recorded similar results obtained in studies of garcinia indica fat (kokum butter), stillingia tallow and piqua fat.

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THE INTESTINAL ABSORPTION OF FATS

Sune Bergström and Bengt Borgström

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THE INTESTINAL ABSORPTION OF FATS

Sune Bergström and Bengt Borgström

I. Introduction

This review covers mainly the different phases of the intestinal absorption of triglycerides and their split products, whereas the absorption of phosphatides, steroids, etc., has been omitted. The absorption of hydrocarbons has been treated due to its importance for the development in this field. We have also omitted those phases of the absorption of triglycerides and fatty acids that have been covered by Deuel (1953) in the second volume of this series. Relatively few references have been made to the extensive clinical literature on fat absorption in humans. The reason for this is that we feel that this field is more advantageously reviewed in a year or two when more unequivocal data with isotopes, etc., have accumulated. The emphasis in the review has been placed on the experimental facts that have accumulated during recent years and older work will only be referred to in connection with specific experimental results. However, a short review of the historical development in this field is given in the first section where references to surveys of older work can be found.

The historical development can conveniently be followed in the different editions of O. Hammarsten's *Lehrbuch der Physiologischen Chemie.*

II. Some Aspects of the Earlier Development

During the latter part of the last century the general views expressed in the text books were that fat was emulsified in the intestine and this emulsion passed into the lymphatics more or less directly. The pancreatic juice was only thought to split a small part of the triglycerides and the fatty acids that were set free were assumed to form soaps that together with the bile stabilized the emulsion (Hoppe-Seyler, 1877). These opinions were mainly based on the visual observation of the content of the intestine and the lymphatics after a fat meal. Chemical evidence was supplied especially by Munk and collaborators (1880, 1891) who showed that triglycerides increased in the lymph after ingestion of triglyceride, free fatty acid or amyl esters of long-chain fatty acids (*cf.* section V(d)).

These views were forcefully disputed by Pflüger (1900) who, in a series of papers, claimed that a total hydrolysis took place and that the fatty acids were absorbed as soaps. Experiments by Henriques and Hansen (1900) in which they fed mixtures of glycerides and hydrocarbons and recovered only the

hydrocarbons in the faeces were also considered as evidence that glyceride molecules had to be split before absorption (*cf.* section VI).

The PFLÜGER theory gained ground and was modified and extended by VERZAR and others during the following decades (see VERZAR and MCDOUGALL, 1936). When it was found that the acid reaction of the upper small intestine would allow the formation of soaps of only a small part of the liberated fatty acid, the importance of the bile acids was stressed (*cf.* SCHMIDT-NIELSEN, 1946).

The solubilizing effect of the bile was for a long time ascribed to the formation of the "choleic acids" of WIELAND and SORGE in spite of the fact that the conjugated bile acids, which do not form choleic acid, are the only ones that normally occur in bile, *cf.* CORTESE and BAUMAN (1936), FIESER and FIESER (1949).

It was later shown, however, that hydrocarbons can be absorbed from the intestine in sizable amounts (EL MAHDI and CHANNON, 1933). This process was further studied by STETTEN (1943) who also showed that they are extensively metabolized in the body.

This evidence and other observations led several authors to propose again that a total hydrolysis was not a necessary prerequisite for absorption of triglycerides (MELLANBY, 1927; DRUMMOND, BELL, and PALMER, 1935; *cf.* RAPER, 1949).

In 1938 FRAZER proposed the "Fat-fatty acid partition theory" and this theory was then extended in many publications during the following decade. It was originally mainly based on chylomicron counts and histological observations after feeding olive oil and free oleic acid containing Sudan dye. The results were interpreted to show that glycerides after emulsification were absorbed via the lymphatics and deposited in the fat depots, whereas ingested free oleic acid was absorbed via the portal vessels and deposited in the liver.

This "partition theory" was then extended and some of the main points of the theory up to about 1950 can be summarized as follows (*cf.* FRAZER, 1940, 1943, 1944, 1945, 1946, 1948, 1949, 1951).

In the lumen of the small intestine the glycerides are hydrolyzed only to a small extent yielding some free fatty acids and lower glycerides (*cf.* ARTOM and REALE, 1935). It was believed that pancreatic lipase could not effect a total lipolysis (FRAZER and SAMMONS, 1945). It was found by FRAZER, SCHULMAN, and STEWART (1944) that bile acids, free long-chain fatty acids and monoglycerides gave better emulsification *in vitro* than any two of these substances. This system was believed to be responsible for the emulsification of the unsplit fat in the intestine to a particle size below 0·5μ. These negatively charged particles were then believed to pass into the mucosa cells through the "cannals" of BAKER (1942) (*cf.* section VII) and then pass unchanged into the lymph except for an exchange of the surface layer.

It was concluded that "Fatty acid passes by the portal vein to the liver while neutral fat goes by the lymphatic route to the systemic blood and thence to the depots to be stored for future use. The degree of lipolysis is, thus, a determining factor in the immediate fate of absorbed fat" (FRAZER, 1943) and

further, "It has been claimed that triglycerides are resynthesized in the intestinal cell. There is no satisfactory evidence that this occurs, and the existence of this resynthesis is assumed on the basis of complete hydrolysis" (FRAZER, 1948).

This theory was thus contrary not only to the concept of total splitting of the glycerides proposed by PFLÜGER–VERZAR but also to earlier experimental work as to absorption and fate of free long-chain fatty acids and glyceride synthesis in the intestinal cells. In spite of that, the "Partition theory" has dominated in this field up to about 1950, as much as the PFLÜGER–VERZAR views did in earlier decades. For a recent version *cf.* FRAZER *et al.* (1954).

The development in this field of biochemistry has neither been rapid nor straightforward. One reason has apparently been the difficulties encountered in making experiments allowing an unequivocal interpretation with a process as dynamic as the absorption from the intestine and the development has sometimes been more dominated by personal opinions than by direct facts. Another factor is that a normal intestinal fat absorption is dependent on a normal intestinal motility that is not present in anaesthetized animals, intestinal loops, etc., that have been used by most earlier investigators.

From about 1950 a rapid development has taken place in this field due largely to the application of new methods. In this way results have been obtained that have made possible unequivocal interpretations and widened our knowledge of many basic mechanisms of the intestinal fat absorption.

The process has turned out to be so dynamic and many-phased that it cannot be covered by any one theory.

III. RECENT DEVELOPMENTS IN METHODS

a. Animal technic

Experiments in which the lymph from the intestine has been collected have been of fundamental importance in the studies of fat absorption. In the earlier development in this field the investigators were generally limited to obtaining lymph from anaesthetized animals. As the normal fat absorption is dependent on a normal intestinal mobility such experiments have sometimes given incomplete and erroneous results because the intestinal mobility is profoundly influenced by anaesthesia and the lymph flow from the intestine is only a fraction of the normal.

The classical experiments that MUNK (1880) performed on a girl with a spontaneous lymph fistula thus gave better recoveries than the experiments on anaesthetized dogs with thoracic duct fistulas (MUNK and ROSENSTEIN 1891); *cf.* also BLOOR (1913).

The introduction of a technic for the collection of lymph from the intestinal or thoracic duct on unanaesthetized rats by BOLLMAN, CAIN, and GRINDLAY (1948) has therefore been of great importance. According to their procedure a polyethylene cannula is inserted into the main intestinal lymph duct or the thoracic duct from an abdominal approach. The animals are then kept in restraining cages in which they can remain unanaesthetized up to a week or

more. They have a good appetite and get 0·9 per cent sodium chloride to drink. The lymph flow is generally about 50 ml but can exceed 100 ml per day when much fat is fed.

The thoracic duct cannulated rats generally seem to be preferable because animals that have an intestinal duct cannula sometimes develop diarrhoea.

In the generally used technic, the cannula is passed directly through the lateral abdominal wall and the lymph collected into a flask outside the cage. In modifications the tubing is led under the skin to the hind leg or a collecting vessel is introduced into the peritoneal cavity that can be emptied through an opening on the animal's back (TASKER, 1951).

Technics similar to those described above have been developed for unanaesthetized dogs (CAIN, GRINDLEY, BOLLMAN, FLOCK, and MANN, 1947; BOLLMAN, FLOCK, CAIN, and GRINDLEY, 1950; GLENN, CRESSON, BAUER, GOLDSTEIN, HOFFMAN, and HEALEY, 1949) and cats (BERGSTRÖM, BORGSTRÖM, and CARLSTEN, 1954). In the latter work recoveries over 70 per cent were obtained as compared to corresponding experiments on chloralose anaesthetized cats where only a maximum of 12 per cent of the fat was recovered (BERGSTRÖM, BORGSTRÖM, CARLSTEN, and ROTTENBERG, 1950) (*cf.* also ECKSTEIN, 1925; LITTLE and ROBINSON, 1941).

In experiments on rats recoveries of over 90 per cent of the absorbed fat is not uncommon even if there is a considerable variation from animal to animal (*cf.* p. V(d.)). These methods are therefore well suited for studies of both quantitative and qualitative aspects of the intestinal fat absorption.

However, some caution has, of course, to be exercised in interpreting the results. Entirely normal pressure conditions and lymph flow cannot be expected when an open cannula has been inserted in the intestinal lymph duct or the thoracic duct. The animal also loses large volumes of water, protein, salts, etc., that are difficult to replace entirely. These factors probably contribute to the large individual variation that has been recorded by different investigators.

Methods have furthermore been described for the collection of blood from the portal vein from unanaesthetized dogs (DENTON, GERSHOFF and ELVEHJELM, 1953). BIERMAN, STEINBOCH, WHITE, and KELLEY (1952), working with humans, collected blood from the portal vein by a percutaneous, transhepatic approach under local anaesthesia.

b. Analytical methods

The analytical methods treated in this section are only such which have relation to work on the evaluation of the mechanism of the intestinal absorption of fats.

Analytical estimation of the composition of glyceride mixtures—Methods for the complete quantitative analysis of the composition of glyceride mixtures found by hydrolysis of triglycerides with lipase *in vitro* or *in vivo* are now available.

DESNUELLE, NAUDET, and CONSTANTIN (1951) have calculated the composition of mixtures formed during hydrolysis of triglycerides with pancreatic

lipase *in vitro* and *in vivo* by estimating the amounts of free fatty acids, total fatty acids, free glycerol, combined or glyceride glycerol and 1-monoglycerides. The 1-monoglyceride and combined glycerol are determined with a modification of the periodic acid method of POHLE and MEHLENBACHER (1950). The method is, however, not generally applicable unless the composition of the monoglyceride mixture formed is known because 2-monoglycerides do not react with periodate. A method for the analysis of the amounts of 1- and 2-monoglycerides present in a glyceride mixture has been proposed by MATTSON, BENEDICT, MARTIN, and BECK (1952). According to this procedure the 1-monoglycerides are assayed by the periodic acid method in one sample and in another sample the total 1-monoglycerides after conversion of the 2-monoglycerides to the 1-isomer. As catalyst for the isomerization MATTSON *et al.* (1952) used perchloric acid which under the given conditions gave about 90 per cent conversion of the 2-isomer. The incomplete isomerization obtained makes the use of a conversion factor necessary. Heat also causes a conversion of 2-monoglycerides to 1-monoglycerides but the variations in the extent of isomerization and accompanying hydrolytic and esterification reactions make this method unsuitable. The presence of minute amounts of ferric chloride seems, however, to catalyse the heat isomerization so that it can be of practical use. The extent of isomerization obtained with this method is about the same as that obtained by perchloric acid method (BORGSTRÖM, 1953). The incompleteness of the isomerization seems to be due to the fact that under the influence of the catalysts an equilibrium mixture containing about 90 per cent 1-monoglyceride is formed (MARTIN, 1953b). Because of the instability of the 2-monoglycerides caution must be taken when handling glyceride mixtures to be analysed for 2-monoglyceride content.

Because the periodic acid reagent gives values for apparent 1-monoglyceride in fats known to be free of 1-monoglycerides the estimation of the small amounts of monoglycerides present in glyceride mixtures of most biological material is not very accurate with this procedure. For more exact values it is preferable to isolate the monoglyceride fraction from the tri- and di-glycerides before using the periodic acid method (BORGSTRÖM, 1954). If the monoglyceride content is known the amounts of tri- and di-glycerides can also be calculated from the hydroxyl value of the mixture (MATTSON, BENEDICT, MARTIN, and BECK, 1952). The composition of a glyceride mixture can also be determined gravimetrically after the separation of the mono-, di- and tri-glycerides by chromatography.

Separation and isolation of different lipids—The need for methods enabling the quantitative separation of the different constituents of lipid mixtures on a small scale is apparent, especially in work with isotopes. Here the application of adsorption and partition chromatography has been of the greatest importance.

Isolation of phospholipids—This topic has recently been fully treated in this series by DESNUELLE (1952). Since then a method has appeared for the separation of phospholipids from neutral lipids and fatty acids by chromatography using silicic acid (BORGSTRÖM, 1952). When a total lipid extract of animal origin in chloroform solution is passed through a column of silicic acid, neutral lipids and free fatty acids pass through the column while the phospholipids

are retained and later can be eluted with chloroform-methanol mixtures. The separation obtained with this method is for many purposes superior to that obtained by the acetone precipitation method. A modification of this procedure has been developed by FILLERUP and MEAD (1953).

Isolation of fatty acids—Free fatty acids can effectively be separated from neutral lipids by extracting the solution in light petroleum with alkaline 50 per cent aqueous ethanol. However, if monoglycerides are present the use of the ion exchanger IRA–400 has proved a valuable alternative to this separation

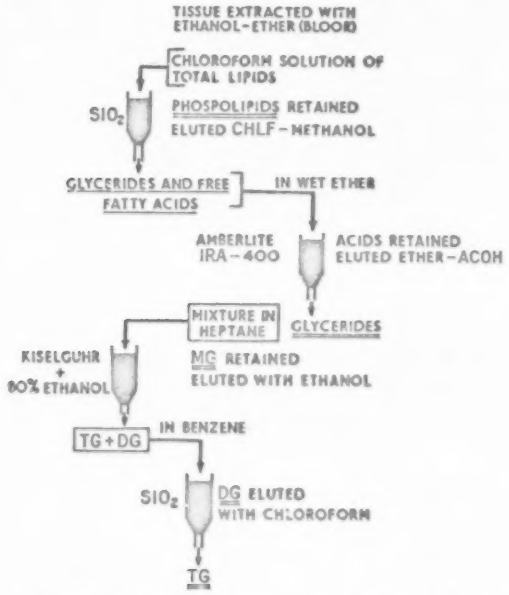

Fig. 1. Separation of a mixture of triglycerides (TG), diglycerides (DG), monoglycerides (MG), phospholipids and free fatty acids (BORGSTRÖM, 1952a, 1954a).

(CASON, SUMRELL and MITCHELL, 1950; BORGSTRÖM, 1952b). A solution of the mixture in wet ether is passed through the ion-exchanger IRA–400 (OH-form). All neutral glycerides pass through whereas free fatty acids are retained and can be eluted by ethanol-hydrochloric acid or preferably ether-acetic acid 9 : 1. Monoglycerides are not isomerized during the passage of the column of IRA–400 (OH) (BORGSTRÖM, 1954). FILLERUP and MEAD (1953) have separated free fatty acids from triglycerides with silicic acid columns.

Isolation and separation of tri-, di- and monoglycerides—ZILCH and DUTTON (1951) investigated the possibility to separate different fatty acid esters by counter-current distribution and found that mono- and triglycerides could be separated using 80 per cent aqueous ethanol and heptane as solvents. Modifications of this method have later been used by KUHRT, WELCH, BLUM, PERRY, and WEBER (1952) and by MATTSON, BENEDICT, MARTIN, and BECK (1952) using counter-current distribution and by BORGSTRÖM (1954) using partition

chromatography. With these methods monoglycerides can be effectively separated from diglycerides, triglycerides and free fatty acids. Separation of tri- from diglycerides and 1-mono- from 2-monoglycerides is not possible with these solvent systems.

A method for the complete separation of tri-, di-, and monoglycerides has been worked out using silicic acid as adsorbent (BORGSTRÖM, 1954). It has been found, however, that 2-monoglycerides partly are isomerized to 1-monoglycerides during the passage through the silicic acid column. If the original monoglycerides are wanted they must be separated from the di- and triglycerides by counter-current distribution or partition chromatography.

A method for the separation of 1- and 2-monoglycerides has been published by BORGSTRÖM (1954). According to this procedure the monoglyceride mixture is oxidized with periodic acid that splits the glycol group in the 1-monoglycerides. The oxidation product of the 1-monoglyceride can then be separated from the unchanged 2-monoglyceride by chromatography on silicic acid. With this method the 1-monoglyceride cannot be recovered in the unchanged form but when the separation of the fatty acids of 1- and 2-monoglycerides is wanted the method can be used. So far these methods have only been used for the separation of glycerides of long-chain fatty acids.

Isolation of glycerol—In work with carbon labelled glycerol the isolation of the glycerol activity has been achieved by oxidation of the glycerol with periodic acid and precipitation of the formed formaldehyd with dimethyldihydroresorcinol as methylene bismethone (VORLÄNDER, IBLE, and VOLKHOLY, 1929; IONESCU and BODEA, 1930; WEINBERGER, 1931, YOE and REID, 1941; BRICE, SWAIN, SCHAEFFER, and AULT, 1945).

Separation of fatty acids—The methods for the separation of fatty acids are also of importance in this connection as it has been found that different acids are treated differently in several respects during the absorption process. A labelled acid can furthermore be transformed into other acids already during the absorption process in the intestine and the location of the isotope in the different acids therefore has to be investigated.

As a comprehensive review of this field has been published by HOLMAN (1952) in this series only a few recent developments will be mentioned that are of special interest.

BOLDINGH (1948, 1950, 1953) has recently developed a reversed phase partition chromatography in which powdered rubber is used as support for the less polar phase. By varying the water content of the mobile phase, acids of different chain lengths are separated. He has recently used this method for the separation of some saturated and unsaturated acids. In this chromatography the introduction of one double bond into a saturated acid changes the properties so that the acid appears together with the saturated acid with two carbon atoms less, i.e. oleic acid behaves like palmitic acid. When the acids in this band then are hydrogenated and rechromatographed the stearic acid that has been formed from oleic acid is clearly separated from the palmitic acid originally present. BOLDING's methods have been extensively used in absorption

studies on humans (FERNANDES, 1953; FERNANDES, WEIJERS, and VAN DE KAMER, 1953).

A wider choice of solvent systems can be used in the other type of reversed phase partition chromatography that was introduced by HOWARD and MARTIN (1950) in which kiselguhr (filter aid), rendered hydrophobic with silanes, was used as support for the less polar phase. This method has also found extensive use for the separation of free and conjugated bile acids (BERGSTRÖM and SJÖVALL, 1951; SJÖVALL, 1953; NORMAN; 1953).

JAMES and MARTIN (1952) recently introduced the "vapour phase chromatography" of fatty acid esters up to C_{12}. This method has also been extended up to C_{22} acids by CROPPER and HEYWOOD (1953).

NIJKAMP (1951, 1953) has modified his earlier method for the separation of C_4 to C_{10} acids to effect the separation of C_{10} to C_{20} acids with the solvent system methanol-*iso*-octane on silica-gel tinted with bromothymol blue.

AHRENS and CRAIG (1952) have used counter-current distribution for the separation of higher fatty acids.

Since labelled unsaturated acids are now used, the need for methods for the separation of these acids from one another and from saturated acids on a small scale have increased.

The classical lead salt fractionation in its different modifications has been shown to be very inadequate for these purposes. One method is that referred to above (BOLDINGH, 1953) and a similar method has also been used by POPJAK (1953).

SAVARY and DESNUELLE (1953) have published a method to separate saturated and unsaturated acids after hydroxylation of the latter by permanganate. The separation of the reaction product was made by partition chromatography.

BERGSTRÖM and PÄÄBO (1954) have worked out a method based on the same principle but using performic acid for the hydroxylation and have used ordinary chromatography on silicic acid to separate the methyl esters of saturated acids and the dihydroxy acid formed from oleic acid.

Isolation of cholesterol and cholesterol esters—Cholesterol and cholesterol esters can conveniently be separated by chromatography on alumina or silicic acid (TRAPPE, 1940; HESS, 1947; BORGSTRÖM, 1952b). Cholesterol esters can also be separated from glycerides on these absorbents but if alumina is used, it has been found to be impossible to recover the glycerides quantitatively as they are partially hydrolyzed on the column (TRAPPE, 1940; KATHEN, 1950). With silicic acid as adsorbent no such troubles interfere with the elution of the glycerides (TRAPPE, 1940; BORGSTRÖM, 1952; FILLERUP and MEAD, 1953).

c. Labelled compounds

Fatty acids—The first isotopically labelled fatty acids used in metabolic studies were obtained by partial reduction of linseed oil with deuterium gas (SCHOENHEIMER and RITTENBERG, 1933). Similar methods have since been used in many investigations. The exchange of carbon-bound hydrogen against deuterium with D_2SO_4 or in D_2O in the presence of alkali and active platinum results in α-labelling or random labelling, respectively (HEYNINGEN, RITTEN-

BERG and SCHOENHEIMER, 1938; *cf.* recent paper by BELL *et al.*, 1953). The method to secure deuterium labelled fatty acid from animals fed deuterium oxide (*cf.* RITTENBERG and SCHOENHEIMER, 1937) has also been used by several authors.

Carbon labelled fatty acids can conveniently be prepared from labelled acetate by feeding it to rats or mice or with liver slices. Saturated fatty acids carbon labelled by chemical means have mostly been labelled in the carboxyl group by the usual technic of treating the appropriate bromide with labelled cyanide and hydrolyzing the resulting nitrile or by treating the Grignard complex with labelled carbon dioxide (*cf.* CALVIN *et al.*, 1949; CROMPTON and WOODRUFF, 1950).

Palmitic acid-6-^{14}C has been prepared by DAUBEN (1948). Several saturated carboxyl labelled acids are now available commercially.

Oleic acid-1-^{14}C has been prepared by BERGSTRÖM, PÄÄBO, and ROTTENBERG (1952) by the silversalt-bromine degradation of erythro-9,10-dihydroxy-stearic acid followed by a nitrile synthesis and a stereospecific elimination of the hydroxyl groups according to BOWMAN and AMES (1951) with formation of the *cis*-9,10 double bond.

The decarboxylation and reformation of linoleic acid has been carried out *via* the tetrabromo acid with a Grignard reaction on the nor-bromide by HOWTON, DAVIS, and NEVENZEL (1952). A reaction sequence with a nitrile-synthesis with the key-step being the differential solvolysis of the primary bromine atom of the nor-penta bromide has also been worked out (BERGSTRÖM, PÄÄBO, and ROTTENBERG, 1954).

The general route for synthesis of the pure *cis*- or *trans*-isomers of monoethylenic fatty acids has been worked out by AMES and BOWMAN (1951). In combination with Kolbe—or "anodic" synthesis—practically any *cis*- or *trans*-mono-unsaturated acid with labelling at any specific atom can now easily be prepared (*cf.* WEEDON, 1952; BOUNDS, LINSTEAD, and WEEDON, 1953).

Similarly the utilization of acetylenic intermediates for the synthesis of unsaturated fatty acids with *cis*-double bonds (AHMAD and STRONG, 1948) has recently been extended to several methods for the synthesis of *cis,cis*-linoleic acid (RAPHAEL and SONDHEIMER, 1950; GENSLER and THOMAS, 1951; WALBORSKY, DAVIS, and HOWTON, 1951) that allow specific labelling of this acid in any position.

Glycerol—Deuterium labelled glycerol has been prepared by FAVARGER, COLLET, and CHERBULIEZ (1951) by reduction of dihydroxyacetone by sodium amalgam in deuteriumoxide and by BERNHARD and WAGNER (1952) by partial hydrogenation of propargyl alcohol with deuterium to allyl alcohol and hydroxylation of the latter to glycerol.

Glycerol-1-^{14}C has been prepared by DOERSCHUK (1951) from ethyl glycerate-1-^{14}C by reduction with lithium aluminium hydride. SCHLENK and DEHAAS (1951) prepared glycerol-1,3-$^{14}C_2$ by condensing paraformaldehyde-^{14}C and nitromethane followed by reduction and diazotation. KARNOVSKY and GIDEZ (1951) have outlined a synthesis starting from labelled acetic acid.

Glycerol-^{14}C is now available from several commercial sources.

Labelled glycerides—For the synthesis of labelled tri-, di- or mono-glycerides several standard methods are available (*cf.* BREUSCH, 1950; DEUEL, 1953).

Labelled triglycerides have been prepared by several methods (DAUBEN, 1948; GEYER, MATTHEWS, and STARE, 1949; BERGSTRÖM, BLOMSTRAND, and BORGSTRÖM, 1954).

A simple way to prepare a triglyceride randomly labelled with saturated acid is to make a transesterification of an unlabelled triglyceride with the labelled acid with sulphuric acid as catalyst (BERGSTRÖM, BORGSTRÖM, and ROTTENBERG, 1952). Under these conditions an exchange takes place both at the primary and secondary positions (BORGSTRÖM, 1954).

SCHLENK, LAMP, and DEHAAS (1952) have described a method for the synthesis of glycerides in which the alcoholic groups of the glycerol successively are built up, beginning from palmitoxyacetic acid. In this way the alcoholic groups of the glycerol become available to react with individual fatty acids and the possibility is given to build up glycerides in which the position of the fatty acids with respect to labelled carbons of the glycerol will be known.

2-Mono- and 1,2-diglycerides containing unsaturated fatty acids have not been available until recently. The preparation of unsaturated 2-monoglycerides has been achieved by splitting off the benzylidene group of 1,3-benzylidene 2-acyl glycerol with boric acid (MARTIN, 1953). A 1,2-diglyceride containing unsaturated fatty acids has been prepared biosynthetically by BORGSTRÖM (1953). No method for their chemical synthesis has so far been described.

Bile acids—The common bile acids labelled with ^{14}C in the carboxyl group have been prepared *via* the norbromides followed by a nitrile synthesis (BERGSTRÖM, ROTTENBERG, and VOLTZ, 1953) and a new procedure has been used for the preparation of the glycine and taurine conjugates (BERGSTRÖM and NORMAN, 1953; NORMAN, 1955).

IV. HYDROLYSIS OF TRIGLYCERIDES BY PANCREATIC LIPASE *in vitro*

a. Pancreatic lipase

Our knowledge of the lipid splitting enzymes of the pancreatic juice and intestinal secretion is still very incomplete. Since the work of WILLSTÄTTER and WALDSCHMIDT-LEITZ (1923) only very few attempts have been reported to purify the pancreatic lipase. GYOTUKO and TERASHIMA (1930) showed that lipase can be precipitated with 55–60 per cent ammonium sulphate but that at the same time a large part of the lipase activity is lost. GLICK and KING (1933) found that pancreatic lipase behaves as a globulin as it is best extracted with 10 per cent sodium chloride solution from dried fat-free pancreatic powder and is precipitated by half saturated ammonium sulphate and saturated magnesium sulphate. A rapid loss of activity was observed when these preparations were dialysed or dried. By precipitation with magnesium sulphate GLICK and KING obtained a purification of 10·7 times based upon lipase units per milligrammes of nitrogen.

Baumann and Laeverenz (1934) claim to have obtained a crystalline lipase protein but give no data which evidence the purity or identity of the substance.

Byrne, Phinney, Schachter, and Young (1951) seem to be the first to try to obtain data for the distribution of the different enzymes of the pancreatic juice after electrophoresis. Using diethylbarbiturate buffer, ionic strength 0·12 and *p*H 8·6 they found at least six peaks by free electrophoresis of canine pancreatic juice and concluded that lipase activity might be associated with a protein fraction of medium mobility. The result of a similar investigation by Grossberg, Komarow and Shay (1952) showed the lipolytic activity of canine pancreatic juice to be associated with two components of low mobilities using the same buffer and *p*H but an ionic strength of 0·1.

b. Rate and extent of hydrolysis

For a long time it has been assumed that the hydrolysis of long-chain glycerides comes almost to a stop *in vitro* when only some 30 per cent of the glycerides is hydrolysed (Frazer, 1951) unless calcium ions are present in sufficient amounts (Balls, Matlack, and Tucker, 1937). In these earlier experiments the lipase sources generally have been extracts or suspensions of dried pancreas and the course of the hydrolysis *in vitro* in the presence of bile has been characterized by a rapid initial hydrolysis of 20 to 30 per cent of the triglyceride. Increasing the time or the amount of enzyme preparation manifold has not increased the extent of hydrolysis (Frazer and Sammons, 1945).

Using a mixture of pancreatic juice and bile as obtained from a cannula inserted into the lower part of the bile-pancreatic duct of rats, Borgström (1952) obtained a nearly complete hydrolysis of corn oil *in vitro* at an initial *p*H of the mixture of 6·4.

Effect of calcium ions—Willstätter, Waldschmidt-Leitz, and Memmen (1923) assumed that the accelerating effect on the pancreatic lipolysis of added calcium ions was due to the formation of calcium soaps which formed complex adsorbates between the fat and the lipase. They found no accelerating effect of calcium ions at acid *p*H values. Experiments by Schönheyder and Volqvartz (1945) showed that the addition of calcium soaps of oleic, lauric and stearic acid to the corresponding triglycerides was without any activating effect, while the addition of calcium salts accelerated the hydrolysis on both the acid and alkaline side. They further found that in the case of triglycerides with fatty acids with 10 or more carbon atoms the activation produced by added calcium ions is more pronounced in the most acid range. They interpreted these results as an indication that the activating effect of calcium ions towards the higher triglycerides in the acid medium is essentially due to the removal of strongly inhibiting fatty acids as calcium soaps. That calcium ions activate the pancreatic lipolysis in both acid and alkaline media has later also been found by Borgström (1954). In these experiments it was also found that calcium ions increased the rate of hydrolysis of glycerides by pancreatic lipase and decreased the rate of resynthesis of glyceride ester bonds (see section IV(d)).

Influence of some other substances—Tween: Minard (1953) has shown that

Tweens, which are fatty acid esters of polyoxyethylene sorbitan, strongly inhibit the action of pancreatic lipase on a corn oil emulsion *in vitro*. Tween 80 was found the most inhibitory followed by Tweens 60 and 20. In the same paper it is reported that the inhibitory effect of Tweens on the pancreatic lipolysis *in vitro* is reversed by the addition of bile acids. MINARD was of the opinion that Tweens effect an inhibition reaction in which a less reactive substrate inhibits enzyme action upon a more reactive substrate.

As this investigation on the inhibitory effect of Tweens on the pancreatic lipolysis was undertaken with a *p*H value of 8·5 in the medium, the importance thereof under physiological conditions (*p*H 6 to 7) cannot be judged at present.

Peptides and amino acids—Experimental results have been reported indicating an activating effect of amino acids and peptides in pancreatic lipase. WILLSTÄTTER and MEMMEN (1923) observed a small increase in the rate of hydrolysis of tributyrin on the addition of alanine and leucylglycylglycine in alkaline media. DAWSON (1927) found that a series of different amino acids accelerated the hydrolysis in alkaline or neutral but not in acid solution of ethyl butyrate and olive oil by pancreatic lipase. This effect was assumed to be due to an increase of the stability of the enzyme. YAMAMATO (1951) reported that several amino acids and some organic acids accelerate the hydrolysis of olive oil and triacetin by pancreatic lipase in alkaline solution. Histidine was found to be especially active and reactivated lipase solution after dialysis (YAMAMATO, 1951b).

Ketones, aldehydes, alcohols—MURRAY (1929) found that substances containing a carbonyl group produce an inhibition of the velocity of pancreatic lipolysis. Some secondary alcohols were also found to have an inhibitory effect. Similar results were obtained by WEINSTEIN and WYNNE (1935) and it was concluded that the carbonyl group forms some sort of attachment with reactive groups in the lipase molecule. The inhibitory effects of secondary alcohols were supposed to be the effect of a reversal of hydrolysis (MURRAY, 1929).

Influence of p*H*—The values given in the earlier literature for the *p*H optimum of pancreatic lipase vary widely apparently depending on the method used for the activity determination. Many of the recorded data are based on titration values which cannot be expected to represent the initial velocity. The substrate used and also the buffer system seem to effect the *p*H optimum (WEINSTEIN and WYNNE, 1935).

The first more detailed study undertaken of the *p*H activity curve of pancreatic lipase originates from SCHÖNHEYDER and VOLQVARTZ (1945). Using glycerol extracts of pig pancreas they estimated the influence of *p*H on the initial reaction velocity of different triglycerides in the absence of bile and calcium ions. From these experiments it appears that the *p*H optimum is displaced to a higher *p*H value with increasing number of carbon atoms in the fatty acids of the glyceride; from about 7 for triacetin to 8·8 for tristearin.

With rat pancreatic juice lipase the *p*H optimum with olive oil as substrate was found to be just above 8 (BORGSTRÖM, 1954).

Influence of bile acids—The bile acids generally have been assumed to be activators for pancreatic lipase in alkaline media (WILLSTÄTTER, WALDSCHMIDT-LEITZ, and MEMMEN, 1923; SHODA, 1926; HOLWERDA, 1938; ROTHLIN and SCHALCH, 1944; MINARD, 1953). In acid media both activating (SHODA, 1926) and inhibitory effects (WILLSTÄTTER, WALDSCHMIDT-LEITZ, and MEMMEN, 1923; HOLWERDA, 1938) on the pancreatic lipolysis have been reported. In these investigations the enzyme source generally has been glycerol or water extracts of dried pancreatic glands and the bile acids added have been in the form of dehydrated ox bile or as bile acids prepared from bile.

Using synthetic taurocholic acid and rat pancreatic juice as enzyme source BORGSTRÖM (1953) has found an activating effect of taurocholic acid on the

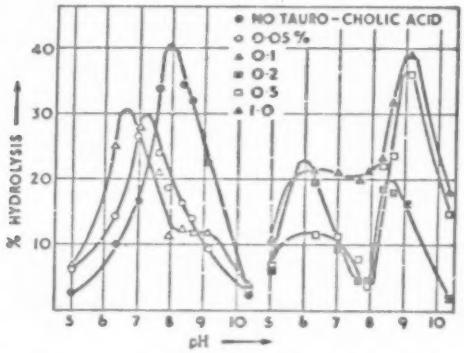

Fig. 2. The influence of different concentrations of sodium taurocholate on the initial rate of hydrolysis of olive oil by rat pancreatic juice (BORGSTRÖM, 1954).

pancreatic lipolysis in acid media with olive oil as substrate. While the *p*H optimum in the absence of bile acids was found to be slightly above 8, taurocholate in concentrations up to 0·2 per cent gradually displaced the *p*H optimum to about *p*H 6 or slightly less. At higher concentrations of taurocholate a new *p*H maximum appeared near *p*H 9.

As the taurocholate concentration in the small intestine of the rat seems to be in the region that displaces the optimum of the pancreatic lipase from 8 to 6–6·5, i.e. the *p*H that is prevailing in the upper small intestine, this effect may be one of the important physiological functions of the bile acids in fat digestion. This investigation should be extended to enzymes and bile from other organisms.

The common concept that soaps cannot exist at the *p*H of the content of the upper part of the small intestine apparently has to be modified after the work of SCHMIDT-NIELSEN (1946). When electrometric titrations were undertaken with oleic acid it was found that the fatty acid-soap system could not be treated in the usual manner in calculations of the dissociation constants. From the titration curves obtained SCHMIDT-NIELSEN calculated that in a pure oleic acid system about 14 per cent of the acid was present as soap at *p*H 7 and 5 per cent at *p*H 6. If dodecanesulphonic acid, which is known to solubilize fatty

acids, was added, the corresponding fractions were calculated to be about 25 and 10 per cent respectively. As the bile acids also are known to form micelles they can be supposed to act as the paraffin sulphonic acids which would mean that a considerable proportion of long-chain fatty acids is present in the intestinal content in the form of soap micelles.

In this connection it might be mentioned that DASHER (1952) has studied the surface activity of bile salts, fatty acids and monoglycerides in mixtures simulating that occurring during fat absorption.

Determination of lipase activity—As a detailed survey on this subject recently has appeared by AMMON and JAARMA (1950) it shall only be treated shortly here.

As the presence of esterases in pancreatic extracts has been evidenced (HOFSTEE, 1952) the use of glycerides of long-chain fatty acids must be considered most appropriate as substrate for lipase determinations in pancreatic juice and extracts. During recent years a tendency to use emulsions of olive oil or corresponding triglycerides as substrate for the lipase determinations is apparent. As emulsifiers phosphatides (MINARD, 1953) and gum arabic (HOFSTEE, 1952) have been used. As the Tweens inhibit the pancreatic lipase, their use as emulsifiers is inappropriate (MINARD, 1953). The addition of calcium ions has been found to be of value as they promote a linear relationship between the amount of enzyme and hydrolysis over a much wider range than in the absence of calcium ions (MINARD, 1953).

A preliminary report of a rapid and sensitive phototurbidometric method for lipase determination using a natural fat emulsion as substrate has recently been published by KOMAROV, SHAY, and ZISLER (1953). Tweens have been reported as substrates for lipase activity determinations (GOMORI, 1945; ARCHIBALD, 1946; BOISSONNAS, 1948).

c. *Formation of lower glycerides*

The formation of intermediary products during the hydrolysis of triglycerides by pancreatic lipase *in vitro* was first demonstrated by ARTOM and REALE (1935). These authors digested olive oil with extracts from dog pancreas and estimated the acetyl value by the method of WEST, HOOGLAND, and CURTIS (1934) of the mixture obtained after partial hydrolysis. The hydroxyl values obtained (12·1 to 104·2) evidenced the presence of partial glycerides but gave no indication of the composition of the glyceride mixture. (Theoretical hydroxyl value for mono-, di-, and triglycerides are 315, 90, and 0.)

BALLS, MATLACK, and TUCHER (1937) could find no evidence for the formation of partial glycerides during hydrolysis of tristearine *in vitro* by pancreatic lipase in the presence of calcium ions but results of later investigators have confirmed the findings of ARTOM and REALE (1935). The formation of partial glycerides during pancreatic lipolysis *in vitro* was confirmed by FRAZER and SAMMONS (1945) also using acetyl value determination. They also reported the isolation of monoglycerides from *in vitro* digests as phthalic acid derivatives.

Detailed studies of the formation of partial glycerides during the hydrolysis

of triolein *in vitro* by pancreatic extracts and in some cases dog pancreatic juice under different conditions have been performed by DESNUELLE, NAUDET, and ROUZIER (1948) and DESNUELLE, NAUDET, and CONSTANTIN (1950, 1951).

At *p*H 7 or 8 and in the presence of bile acids prepared from ox bile in a concentration of 0·19 per cent but in the absence of calcium ions they obtained a hydrolysis "génératrice de diglycérides," i.e. the glyceride mixture consisted of triglycerides and only traces of glycerol liberated during 4 hr. If, furthermore, one calcium ion was present for every fatty acid residue the hydrolysis was found to go further and result in an accumulation of monoglycerides. Total hydrolysis resulting in liberation of glycerol was still insignificant (DESNUELLE, NAUDET, and ROUZIER, 1948). Only in the presence of high concentrations of bile acids (1·2 per cent) did they get a hydrolysis "génératrice de glycérol." In these investigations of DESNUELLE *et al.* the hydrolysis of the free glycerides was assumed to proceed at random and no attempts were made to study the configuration of the partial glycerides formed.

SCHÖNHEYDER and VOLQVARTZ (1952) have investigated the action of pig pancreas extracts on tripropionyl glycerol. They found that only 1 mol fatty acid was liberated per mol triglyceride. They assumed 1,2-dipropionylglyceride to be the end product as hydrolysis of synthetic 1,2 and 1,3 dipropionyl glycerol showed that the 1,2 form was not further degraded under these conditions.

BORGSTRÖM (1953) isolated the diglyceride formed *in vitro* by rat pancreatic juice from olive oil by chromatography on silicic acid and demonstrated by oxidation with chromic acid that the diglyceride was of the 1,2-configuration.

As to the further degradation of the diglyceride by rat pancreatic lipase *in vitro* the isolated monoglyceride fraction in the above experiments was found to contain about 20 per cent of 1-monoglyceride. If the 1-monoglyceride was formed directly from the 1,2-diglyceride or by isomerization of the 2-monoglyceride, could not be elucidated from these experiments. The results of other experiments using [^{14}C] fatty acids (*cf.* p. 368) indicate that by pancreatic lipase *in vitro* the 1,2-diglyceride is degraded to a mixture consisting of 10 to 20 per cent 1-monoglyceride and 80 to 90 per cent 2-monoglyceride.

With rat pancreatic juice as enzyme source mixed with rat bile BORGSTRÖM (1952g) obtained a nearly complete hydrolysis of olive oil *in vitro*. In these experiments only negligible amounts of calcium ions were present and even in experiments *in vitro* in which pancreatic juice without bile was used for the hydrolysis a rapid liberation of glycerol occurred.

Thus, in these experiments none of the striking differences in the course of the hydrolysis was found that have been observed by DESNUELLE *et al.* (1948, 1950) depending on the composition of the medium in which the hydrolysis was performed.

The explanation of the differences observed in the above experiments of the course of the hydrolysis by pancreatic lipase *in vitro* is at present not apparent. Possibly the differences in enzyme sources used can be of importance. BORGSTRÖM used rat pancreatic juice as lipase source while DESNUELLE *et al.* used glycerol extracts of pig pancreas but in one case these authors used

canine pancreatic juice. Possibly the relation of amounts of enzyme to substrate can play a role. In the experiments in which DESNUELLE *et al.* used canine pancreatic juice 0·4 ml were used for 1·2 gm triolein as substrate while BORGSTRÖM used 1·25 ml rat pancreatic juice for 0·1 gm olive oil.

d. Formation of new ester bonds during hydrolysis of glycerides by pancreatic lipase in vitro

While the synthetic effect of pancreatic lipase has long been known, its occurrence during physiological conditions has not been demonstrated until recently.

In classical experiments RONA and AMMON (1932) found that in the presence of pancreatic lipase the same equilibrium mixture was obtained starting from n-butyl-butyrate or a mixture of the acid and alcohol. This and other similar experiments made to demonstrate the synthetic effects of pancreatic lipase were generally performed in organic solvents with the alcohol in large excess and the presence of water was found to inhibit the synthesis (*cf.* AMMON and JAARMA, 1950).

FABISCH (1933) was the first to report a synthetic effect of pancreatic lipase

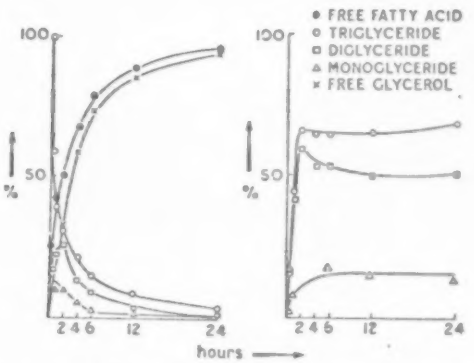

Fig. 3. Hydrolysis of corn oil by rat bile pancreatic juice in the presence of a small amount of labelled palmitic acid. Left part: molar composition (acid/3) at different times. Right part: specific activity of fatty acids of different glycerides in per cent of the activity of the free fatty acids present at each time (BORGSTRÖM, 1954a).

in aqueous emulsions of oleic, palmitic or stearic acid. He used sodium oleate or desoxycholate as emulsifier and added glycerol and pancreatic powder. He then found a decrease of free fatty acids corresponding to at the most 18·5 per cent synthesized glycerides after 5 hr in solutions containing more than 40 per cent glycerol. Glycerides were, however, not isolated.

More definite proof for the formation of new ester bonds by the action of pancreatic lipase under conditions resembling those in the lumen of the small intestine during digestion was supplied by BORGSTRÖM (1952). If a triglyceride mixed with a small amount of a labelled free fatty acid was incubated *in vitro* with a mixture of bile and pancreatic juice of rat, the glycerides became

labelled during the course of the overall hydrolysis (Fig. 3). It was further found (BORGSTRÖM, 1954) that during hydrolysis of triglycerides by pancreatic lipase under these conditions *in vitro* the fatty acids in 1- and 3-position in the glyceride molecule came into equilibrium with the free fatty acids, while

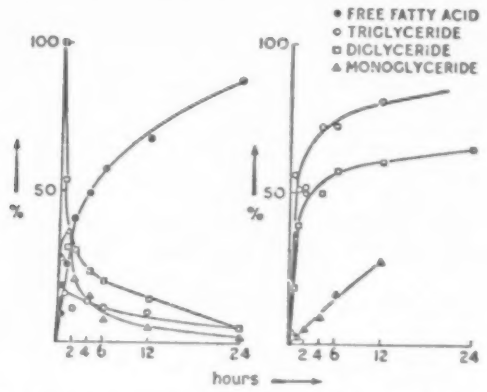

Fig. 4. Hydrolysis of unlabelled diglyceride with rat bile pancreatic juice *in vitro* in the presence of a small amount of labelled palmitic acid. Left part: molar composition (free acids/2). Right part: specific activity of fatty acids of different glycerides in per cent of the activity of the free fatty acids present at each time (BORGSTRÖM, 1954a).

the fatty acids in 2-position were exchanged with free fatty acids much slower. This incorporation of free fatty acids into glycerides during the course of the pancreatic lipolysis is at least partly due to a resynthesis of ester bonds from free

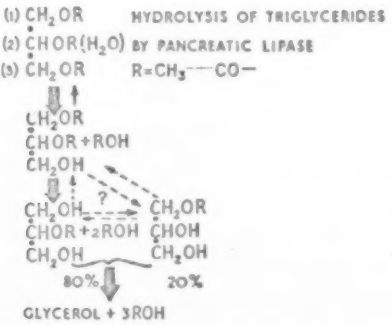

Fig. 5. Scheme of hydrolysis of triglycerides by rat pancreatic lipase. $R = CH_3{\cdot}CH_2 \ldots CO—$.

hydroxyl groups and not only to a transesterification. When a 1,2-diglyceride was incubated under these conditions some labelled triglyceride could be isolated.

However, if some free labelled glycerol was added in the aforementioned experiments no incorporation of the glycerol was observed.

The explanation of these differences is probably that the active centres of the lipase are in contact with the lipid phase or micelle surface at which triglycerides, partial glycerides and free long-chain fatty acids are closely packed. The concentration of water molecules at this region is then relatively low compared to the bulk of the system and the formation of ester bonds from free long-chain fatty acids and the hydroxyl of the partial glycerides can attain a considerable magnitude in spite of the net hydrolysis.

The free glycerol that is distributed in the free water phase does not take part in these reactions nor does butyric acid that presumably also remains in the aqueous phase, whereas stearic, palmitic, oleic, dihydroxystearic and decanoic acid, that form micelles, do.

However, the water-insolubility or the tendency to form micelles is not the only factor determining the incorporation neither *in vitro* and in the intestinal lumen under the influence of pancreatic lipase nor in the intestinal cell. A steric factor must also be of importance as α,α-dimethylstearic acid was not incorporated under *in vitro* conditions (Bergström, Borgström, Trydinc and Westöö, 1954). However, the dimethyl-stearic acid was absorbed and appeared in the lymph both in triglycerides and phospholipids. The formation of glycerol ester bonds in the intestinal cell is thus not a simple reversal of the hydrolysis but most likely proceeds *via* energy rich acyl compounds (*cf.* Kornberg and Pricer, 1953, and p. 382). Likewise cholanic, lithocholic, desoxycholic or cholic acid are incorporated at most in traces under these conditions. Here two steric factors are of importance as both dimethylstearic acid and the bile acids are water insoluble and micelle-forming. The free bile acids are neither incorporated into glyceride ester bonds in the lipase system nor into the glycerides or phospholipids in the cells.

So far the incorporation of acids into glyceride ester bonds in the presence of pancreatic lipase *in vitro* and in the intestinal content has only been observed with medium or long chain fatty acids that allow a relatively close packing in surface films. Various steric influences are clearly of importance.

V. Intestinal Absorption of Triglycerides

a. Composition of intestinal content after feeding fats

Information on the intraluminar phase of fat absorption has been obtained by analyses of the content of the small intestine removed during digestion.

The percentage of free fatty acids found in the lipids recovered from the content of the small intestine during absorption of triglycerides varies widely. Levites (1906) found the percentage free fatty acids of the lipids from the contents of different parts of the small intestine of dog to increase successively from duodenum (28 per cent) to ileum (41 to 66 per cent). Frazer and Sammons (1945) found 30 per cent free fatty acids in the recovered lipids from the small intestine (of 12 rats) 16 hr after the administration of 2 ml olive oil to rats. Borgström (1952) found a mean percentage of 30·3 (17·6 to 42·5) for free fatty acids in the lipids recovered from the small intestine of rats ½ to 3 hr after the

administration of 0·75 ml corn oil. In similar experiments on rats DESNUELLE and CONSTANTIN obtained the figures 15·6 to 24·3 and on two dogs 16·7 and 49·2.

Two to four hours after feeding rats 1·5 ml of a partially hydrogenated vegetable oil MATTSON, BENEDICT, MARTIN, and BECH (1952) found 21·5 to 35·7 per cent of free fatty acids in the lipids recovered from the intestinal content.

By administration to rats of olive oil together with a solution of calcium chloride an increased percentage of free fatty acids of the intestinal content was found by DESNUELLE and CONSTANTIN (1952), viz. 41·5 and 45·8 per cent in two experiments respectively.

After feeding rats corn oil containing 30 per cent free oleic acid BORGSTRÖM (1952g) found as a mean 39·3 per cent free fatty acids in the lipids of the intestinal content. The increase in the percentage of free fatty acids in these experiments, 9·0 per cent, compared to the experiments in which corn oil was fed was found statistically significant and indicates that the rate of absorption of free fatty acids from the small intestine must be limited. The same conclusion was also reached by MATTSON *et al* (1954).

That free fatty acids added in small amounts to triglycerides are absorbed more rapidly than the main bulk of the glycerides has been shown with the use of labelled acids (BORGSTRÖM, 1952d,g, 1953). Fatty acids of different chain length fed dissolved in long chain triglyceride are absorbed with different rates. Short chain fatty acids are absorbed much more quickly than the longer chains are (BORGSTRÖM, 1954b).

The composition of the glyceride mixture obtained from intestinal contents of animals has been studied by DESNUELLE (1951) and DESNUELLE and CONSTANTIN (1952), BORGSTRÖM (1952g, 1954b), MATTSON, BENEDICT, MARTIN, and BECH (1952) and of man by KUHRT, WELCH, BLUM, PERRY, WEBER, and NASSET (1952).

The figures for monoglyceride content given by DESNUELLE (1951) and DESNUELLE and CONSTANTIN (1952) are too low due to their assumption that 2 mol 1-monoglyceride is formed for each 2-monoglyceride. MATTSON, BENEDICT, MARTIN, and BECH (1952) showed that the monoglycerides found in intestinal contents from rats were almost entirely of the 2-isomer. The total monoglyceride content of the lipids recovered from the intestinal contents of rats was found to be 7·5 to 16·4 by MATTSON *et al*. The figures for monoglycerides given by BORGSTRÖM (1952g), 30·9 per cent, were too high due to the unspecificity of the analytical method then used. Figures later obtained by BORGSTRÖM (1954b) for total monoglycerides, 5·4 to 16·5 per cent (mean of seven analyses 9·2 per cent), are in close agreement with the values of MATTSON *et al*.

The values reported by KUHRT, WELCH, BLUM, PERRY, WEBER, and NASSET (1952) for monoglyceride content of intestinal content removed from humans during digestion of a fat rich meal are much higher than those found in the rat. In two experiments these authors found 37·6 per cent and approximately 50 per cent of the recovered lipids to be monoglycerides. In this investigation no attempt was made to determine the composition of the monoglyceride mixture with respect to 1- and 2-monoglycerides.

Values for the content of di- and triglycerides in the lipids of the intestinal contents have been obtained for dogs by DESNUELLE (1951) and for rats by DESNUELLE and CONSTANTIN (1952), MATTSON, BENEDICT, MARTIN, and BECH (1952) and by BORGSTRÖM (1954) by different methods. These values are well in agreement with each other but rather large variations are found from case to case obviously depending on the continuous admixing of unchanged glycerides from the stomach.

In conclusion it must be emphasized, however, that much of the work referred to has been made on fasted animals and with pure lipids. Under most normal conditions triglycerides are only present in the intestine together with larger amounts of other food constituents. Considerable differences were observed when methyl esters were administered to fasted mice alone or when triglycerides or fat-free food was given simultaneously (MEAD, BENNETT, DECKER, and SCHOENBERG, 1951). Systematic studies on the influence of different food constituents on fat absorption are apparently lacking.

b. Incorporation of free fatty acids into glycerides in intestinal lumen

The exchange of glyceride fatty acids and free fatty acids taking place on pancreatic hydrolysis *in vitro* (*cf.* section 4*d*) has also been demonstrated during *in vivo* conditions. If a triglyceride such as corn oil in which a small amount of free labelled fatty acid was dissolved was fed to rats, the glyceride mixture isolated from the intestinal content contained labelled acids (BORGSTRÖM, 1952*g*, 1954*b*). An interpretation of the course and extent of this process *in vivo* is, however, rendered difficult by a continuous admixing of unchanged glycerides from the stomach and of lipids contained in the bile and after secretion to the intestinal content as well as absorption from the intestine. This process, however, initiates the randomization of the fatty acids fed in glyceride which later is continued in the intestinal mucosa (*cf.* section V*d*).

Shorter chain fatty acids such as decanoic acid are incorporated to a smaller extent than long-chain fatty acids in this process and butyric acid fed dissolved in long-chain triglycerides was not at all incorporated into the glycerides in the intestinal content (BORGSTRÖM, 1954*b*).

c. Composition of lipids in mucosa after feeding triglycerides or free fatty acids

During absorption of fat from the small intestine there is a considerable increase of neutral fat and also of free fatty acids that can be extracted from the wall of the small intestine; the amount of phospholipids, however, stays relatively constant (SINCLAIR, 1929; BARNES, MILLER, and BURR, 1941; SCHMIDT-NIELSEN, 1946; BORGSTRÖM, 1952).

Even if the amounts of phospholipids of the small intestinal wall is not changed during fat absorption, there are pronounced changes in their qualitative fatty acid composition depending on the fat being absorbed.

This fact was first demonstrated by SINCLAIR (1929) who found that the

iodine value of the fatty acids of the phospholipids of the small intestinal wall changed with that of the fat fed. This finding led him to postulate that phospholipids were involved as intermediates in the resynthesis of triglycerides. Similar results were later obtained from experiments with elaidic acid and iodized fatty acids. After ingestion these acids were found in high percentage in the intestinal wall phospholipids; after feeding elaidic acid to cats as much as 50 per cent of the small intestinal (mucosa) phospholipid fatty acids was elaidic acid (SINCLAIR and SMITH, 1937). A much smaller incorporation was observed with conjugated linoleic acid (BARNES, MILLER, and BURR, 1941). The early development in this field has been reviewed by ARTOM and PEROTTI (1935) and BLOOR (1943).

Earlier work with labelled phosphate also indicated an increased turnover of the phosphorous part of the phospholipids of the intestinal wall during fat absorption (ARTOM, SARZANA, PERNIER, SANTANGELO, and SEGRI, 1937; PERLMAN, RUBEN, and CHAIKOFF, 1937; FRIES, RUBEN, PERLMAN, and CHAIKOFF, 1938). SCHMIDT-NIELSEN (1946) also observed an increase in the specific activity of phospholipid phosphorous of the small intestine during fat absorption. As a similar increase was not seen after feeding glucose, SCHMIDT-NIELSEN (1946) concluded that the increased turnover of the phospholipids is "really a process of much importance to fatty acid uptake, perhaps essential in it." However, he did not consider the increase sufficient to account for the conversion of all absorbed fat to phospholipids, unless this process was limited to a small fraction of the intestinal wall phospholipids. It is, however, obvious that the phospholipids that can be engaged in fat absorption are those present in the epithelial cells and thus only constitute a minor part of the total phospholipids of the intestinal wall (FAVARGER, COLLET, and VERAGUTH, 1949; SINCLAIR, 1929). Experiments with ^{32}P do not necessarily give any information of the rate of exchange of the fatty acids of the phospholipids and in the original hypothesis of SINCLAIR there is no need for an increased turnover of the phosphorous part of the phospholipid molecules. More recent experiments with labelled saturated acids of different chain length indicate that the intestinal phospholipids have a very rapid turnover of the fatty acid moities, but that there are differences of the extent to which different acids are incorporated into the phospholipids of the intestinal wall. When triolein was fed that had been transesterified with 5 per cent of saturated [^{14}C] fatty acids it was found that the incorporation into the phospholipids of the small intestinal wall was larger the longer the chain (BERGSTRÖM, BORGSTRÖM, and ROTTENBERG, 1952; BORGSTRÖM, 1952). The difference between the metabolic activity of the phospholipids of the entire intestinal wall and the inner part obtained by scraping off the mucosa is also illustrated in this work.

The specific activity of the fatty acids of the mucosa phospholipids rapidly reached 70 per cent of the activity of the mixture. Corresponding value for the phospholipids of the total small intestine only reached about 30 per cent.

In the former case, judging from the rate of the incorporation a very considerable part of the absorbed labelled stearic acid could have been incorporated

into phospholipids during the passage through the intestinal cells from which it was given off into the lymph mainly as triglycerides. FAVARGER *et al.* (1949) have found indications that choline-containing phospholipids incorporated more than the non-choline containing.

Summarizing the evidence so far available concerning the phospholipids in the intestinal mucosa, there is a certain increase of the turnover rate of the phosphate part, but the quantitative significance is not quite clear. There is, however, a very rapid exchange between the fatty acids that are transported through the cells and the phospholipids present and differences between different fatty acids have been observed. There is also a certain amount of net synthesis of phospholipids taking place during fat absorption as labelled phospholipids are given off to the lymph while the amount in the mucosa stays constant. This amount is, however, generally well below 10 per cent of the triglycerides simultaneously given off to the lymph.

FAVARGER (1949) and FAVARGER and GERLACH (1953) have studied the distribution of different D-labelled acids in different parts of the washed small intestine of the rat 3 hr after feeding.

The labelled acids were administered as free acid mixed with different acids or as triglycerides. The highest concentration of the labelled acid in the glycerides was generally found in the upper or middle third of the intestine. Feeding of a labelled triolein led to more isotope in the upper two-thirds whereas labelled tristearin gave the highest concentration in the lowest third.

500 mgm of [D] palmitic acid alone gave an even distribution of labelled palmitic acid throughout the small intestine, but admixtures of elaidin or free oleic acid decreased the incorporation in the lowest third indicating that the absorption in the latter cases is more rapid so that less fat reached the lower part of the small intestine. It has also been shown that the absorption of stearic acid is quantitatively quite different if it is administered in free form, as tristearin or mixed with an unsaturated fat (BUENSOD and SCRIBANTE, 1952).

There are thus as great differences in the rate and extent of absorption of the same acid, depending on the presence of other glycerides or free fatty acids, as there are between different fatty acids when administered in small amounts incorporated into triolein.

Quantitative studies of the physico-chemical behaviour of mixtures of different fatty acids, partial glycerides, glycerides and bile salts in relation also to the action of lipase and the rate of absorption is urgently needed in this field.

d. Composition of lymph and lymph lipids after feeding triglycerides or free fatty acids

The changes in lymph volume, lymph proteins and fat of the lymph from the small intestine during absorption of corn oil have been studied in the rat by BORGSTRÖM and LAURELL (1953).

The lymph water flow from the small intestine increases strongly after the oral administration of fat and it is remarkable that the maximal lymph flow is reached about two hours before the maximal lymph lipid flow. During ab-

sorption of fat the protein concentration of the lymph from the small intestine does not decrease in spite of the increased lymph flow, in contrast to what is seen after the administration of saline solution (SHREWSBURY and REINHARDT, 1952; BORGSTRÖM and LAURELL, 1953).

The electrophoretic protein pattern of the lymph from the small intestine of the rat is practically identical with that of the plasma and shows no differences in fasting or fatty lymph.

The fat particles of fatty lymph which on free electrophoresis move with the albumins (FRAZER, 1949; BORGSTRÖM and LAURELL, 1953) are strongly adsorbed to the paper by paper electrophoresis and thus can be distinguished from the lipid fraction in serum with the same free mobility. The concentration of lipoproteins of plasma type is low in the lymph from the small intestine and no appreciable synthesis of such proteins seems to take place in the mucosal cells of the small intestine during fat absorption.

The main part of the lipids in lymph from fasting and lipid fed animals occurs in a more highly dispersed state than in plasma. The fat particles in intestinal lymph have been shown to flocculate after addition to the lymph of small quantities of protamine (BROWN, 1953) or toluidin blue (LAURELL, 1954), leaving behind a water clear almost fat free solution with no changes in the protein concentration as compared to the original lymph. The proteins of the flocculated fat particles have been studied by LAURELL (1954).

During absorption of glycerides there is a manifold increase of lymph neutral fat and phospholipids, the increase in neutral lipids being greater than that in phospholipids (SULLMAN and WILBRANDT, 1934; BROCKETT, SPIERS, and HIMWICH, 1935; BOLLMAN, FLOCK, CAIN, and GRINDLAY, 1950; BORGSTRÖM, 1952).

After feeding 0·5 ml corn oil glycerides to unanaesthetized rats, the composition of the lymph fat recovered in 24 hr from the main intestinal lymph duct has been found to be approximately as follows: glyceride fatty acids 88 per cent, phospholipid fatty acids 10 per cent, and cholesterol ester fatty acids 2 per cent (BORGSTRÖM, 1952). The distribution of absorbed labelled acids between these lipids is further discussed in section V(*f*.)

Experiments with labelled fatty acids indicate that some of the phospholipid molecules in the lymph from the small intestine during fat absorption are derived from plasma phospholipids (BORGSTRÖM, 1952). Calculation of how much of the lymph phospholipids during fat absorption originates from absorbed fat and from plasma phospholipids respectively is rendered difficult as the incorporation of labelled fed fatty acids into the lymph phospholipids is influenced by the structure of the acids.

That plasma phospholipids can pass capillary walls has been demonstrated by REINHARDT, FISHLER, and CHAIKOFF (1944) who found that thoracic duct lymph of dogs served as a medium for the return to the plasma of a significant part of the phospholipids lost from plasma.

It has long been a well established fact that long chain fatty acids fed as the free acid or as glyceride or esters of other alcohols than glycerol, as ethyl esters, amyl esters, cetyl esters and others, are found in the lymph from the small

intestine exclusively as esters of glycerol and cholesterol (*cf.* MUNK and ROSENSTEIN, 1891; FRANK, 1898; BLOOR, 1912; FREEMAN and IVY, 1935; BORGSTRÖM, 1952). No esters of long chain fatty acids with other alcohols have so far been found in the lymph from the small intestine nor has intestinal lymph been found to contain more than traces of free fatty acids.

Long chain fatty acids fed as such appear in the intestinal lymph of rats as glycerides at a slower rate than when the corresponding amounts of fatty acids are fed as triglyceride (DEUEL, 1953; BORGSTRÖM, 1952). This is at least partly due to a slower emptying rate of free fatty acids from the stomach. As a consequence of this the appearance of fed free fatty acids in the lymph as glycerides is more prolonged than when the corresponding amounts of glyceride are fed. This slower and more prolonged appearance of fat in the lymph after feeding free fatty acids is probably the explanation of the low chylomicron counts observed by FRAZER (1943) after feeding oleic acid to rats compared to when olive oil was fed.

The composition of the fatty acid mixture of the lymph lipids in relation to that of the fed fatty acid mixture will be dealt with in the next section.

If the bile is diverted from the small intestine of rats pronounced changes in the lymph from the small intestine are seen. In the absence of bile in the intestine the fasting lymph, normally opaque, is water-clear with a fat content of only about one-fifth of the normal (BORGSTRÖM, 1953). After feeding labelled fat to bile fistulae rats, only a small percentage of the absorbed activity appears in the lymph (SIPERSTEIN, CHAIKOFF, and REINHARDT, 1952; BORGSTRÖM, 1953) and the lymph volume and lymph fat are only slightly increased, not even reaching the values found for normal fasting lymph. The absence of bile from the intestine apparently not only interferes with the intraluminar phase of fat absorption but also results in a profound change in the metabolism of the intestinal cells.

It is of interest in this connection that when the distribution of bile acids in the normal rat was investigated with [^{14}C] bile acids, more bile acids were found to be present in the intestinal wall than in the liver (BERGSTRÖM, SJÖVALL and VOLTZ, 1953; BERGSTRÖM, ROTTENBERG and SJÖVALL, 1953), i.e. about 10 per cent of the total amount of bile acids present in the body was contained in the wall of the intestine against 2 to 4 per cent in the liver. The remainder was present in the intestinal content.

e. Influence of fatty acid structure on route of absorption

1. *Long chain* ($\geq C_{16}$) *fatty acids*—As mentioned earlier (*cf.* p. 374), good experimental evidence had already been produced during the last century that fed triglycerides or other esters as well as free oleic acid are transported mainly *via* the lymphatics as triglycerides (see especially the classical work of MUNK *et al.*, 1880, 1891).

An increase in lymph glycerides after feeding oleic acid to diabetic as well as normal dogs was also found by FREEMAN and IVY (1935). In *unanaesthetized*

dogs with lymph fistulas BOLLMAN, FLOCK, CAIN, and GRINDLAY (1950) found that feeding either triglycerides or free oleic acid gave rise to a great increase in the lymph glycerides.

Much experimental evidence of a more quantitative nature has accumulated since 1950 to sustain these early views concerning the fate of the long chain fatty acids.

The first investigators to study this problem with the technique of BOLLMAN *et al.* (1948) on unanaesthetized rats were BLOOM, CHAIKOFF, REINHARDT, ENTENMAN, and DAUBEN (1950).

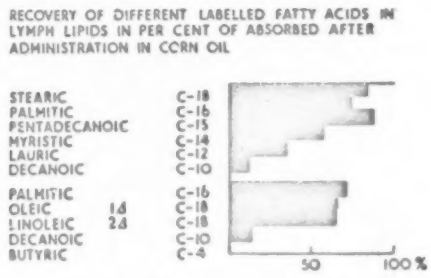

Fig. 6. Recovery of different labelled fatty acids in lymph lipids after administration to rats with lymph fistulas. Upper part from BLOOM *et al.* (1950, 1951), lower part from BERGSTRÖM, BLOMSTRAND, and BORGSTRÖM (1954), BORGSTRÖM (1954).

In these experiments 0·5 ml corn oil containing about 4 mgm of palmitic acid-1-^{14}C as free acid or as tripalmitin was fed by gastric intubation to rats with a thoracic duct or intestinal lymph fistula. In both cases 70 to 92 per cent of the absorbed labelled acid was recovered from the *thoracic duct* lymph and slightly less from the *intestinal* lymph.

In a later paper (BLOOM, CHAIKOFF, REINHARDT, and DAUBEN, 1951), recoveries of up to 97 per cent of the absorbed palmitic acid were reported.

Similar results with different long chain ($\geq C^{16}$) fatty acids on rats with lymph fistulas have been obtained by several authors. REISER (1950) and REISER and BRYSON (1951) used linoleic acid with conjugated double bonds and BORGSTRÖM (1951, 1952) used palmitic and stearic acid-1-^{14}C. Of special interest in connection with earlier work with oleic acid are studies with carboxy-labelled oleic acid (BERGSTRÖM, BORGSTRÖM, and BLOMSTRAND, 1954) and linoleic acid (BLOMSTRAND, 1954).

In work with this technique rather large individual variations between different animals are observed, but the average is generally between 70 and 90 per cent for the recoveries of the long chain fatty acids in the lymph lipids from a fistula in the thoracic duct or the main intestinal lymph duct. One of the reasons for the great variations, especially in the latter case, is undoubtedly the varying anatomical conditions with lymphatico-venous anastomosis, but even with the fistula in the thoracic duct, the individual variations from some rat strains might be partly due to this cause. Several other reasons for these variations are also apparent. A small part of the absorbed acids is already

degraded in the intestinal cells. Furthermore the conditions as to the lymph flow cannot be expected to be quite normal in a fistulated animal even if no clot has formed. It does not appear unlikely that part of the long chain fatty acids not recovered in the lymph is transported from the intestine *via* the portal vessels, but this seems to be only a small amount under normal conditions and it does not appear to have anything to do with the form in which the acid in question has been administered. It seems likely that the animals with the highest recoveries are most nearly approaching the normal state.

Similar results to those on rats, referred to above, have also been obtained in cats by BERGSTRÖM, BORGSTRÖM, CARLSTEN, and ROTTENBERG (1950). Labelled stearic acid was administered as free acid or triglycerides to cats with a thoracic duct fistula who remained anaesthetized during the entire experiment. Low recoveries were obtained but with another technique on unanaesthetized cats recoveries of up to 65 per cent of the labelled acid fed have been obtained (BERGSTRÖM, BORGSTRÖM, and CARLSTEN, 1954).

Extensive data on fat absorption in humans have been obtained by FERNANDES, WEIJERS, and VAN DEN KAMER (1954) and FERNANDES (1953) on a child who developed a spontaneous chylothorax which had to be aspirated through puncture from the time the child was 22 months until it was 30 months old, when the child was cured. The child was each week given a constant diet that only contained one type of fat. The fat in the chyle that was aspirated at the end of each week was then analysed with the methods of BOLDINGH (1950, 1953) and the fatty acid composition of the fat fed and that recovered from the chyle was compared (*cf* Table 1 on p. 381). The fat excretion in the faeces was also followed during the entire period. By selecting fats with widely varying composition, or preparing some synthetically, the absorption of saturated acids C_4 to C_{22} and furthermore oleic, elaidic, linoleic and linolenic acid could be studied. The composition of the chyle fat very closely approximated to that of the fat fed when the abovementioned long chain fatty acids ($\geq C_{16}$), either saturated or unsaturated, constituted the triglyceride fat administered. Small amounts of stearic acid were at least as well absorbed as palmitic acid, but larger amounts were not absorbed so well and led to an increased faecal content of stearic acid. No difference was found in the absorption of oleic, elaidic, linoleic and linolenic acid. This work thus confirms and greatly extends the work of MUNK and ROSENSTEIN (1891) on fat absorption in humans. The results of FERNANDES *et al.* as to short chain acids is mentioned in the following section (p. 379).

That the saturated long chain acids (C_{18} to C_{22}) are only absorbed to a limited extent is well known from earlier work. BERNHARD (1948) has studied the absorption of deuterated behenic acid and it is also indicated that the double bond in erucic acid does not counterbalance the influence of the chain length in this respect (*cf.* also DEUEL, CHENG, and MOREHOUSE, 1947). The influence of fatty acid structure on the rate of absorption and the coefficient of digestibility has been reviewed by DEUEL (1953) in an earlier volume of this series.

The work referred to earlier in this section is thus contrary to that part of the "partition theory" (*cf.* p. 353), according to which fatty acids are absorbed *via* different routes if administered as free acids or as triglycerides. These views were mainly founded on the different chylomicrographic response after feeding oleic acid or olive oil to rats (FRAZER *et al.*, *loc. cit.*). TIDWELL (1950), however,

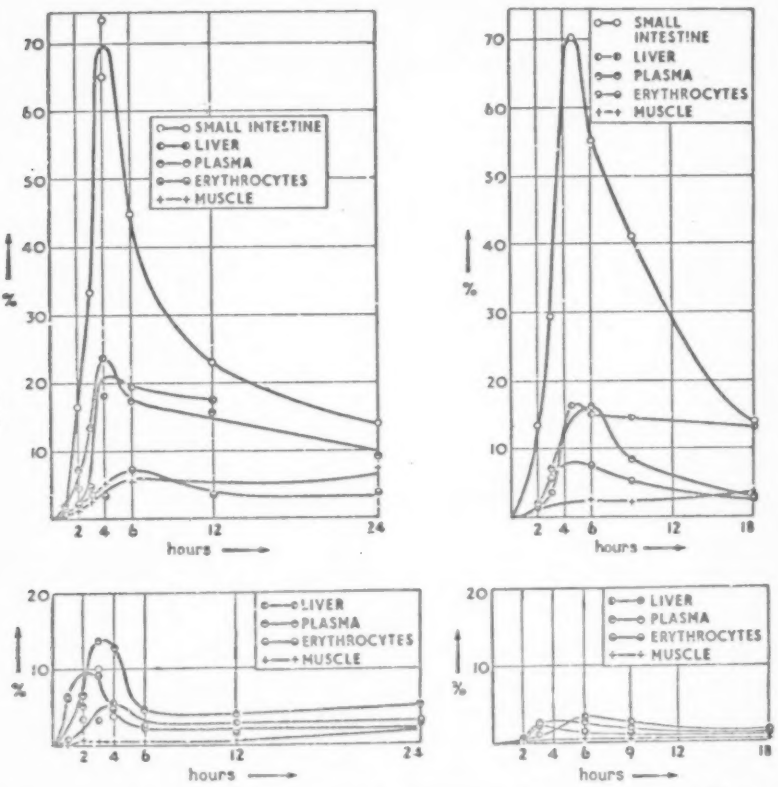

Fig. 7. Specific activity of fatty acids of triglycerides and phospholipids from different organs of rats that had received corn oil (0·1 ml per sq. dm body surface) or corn/oil free fatty acids containing stearic acid-1-^{14}C. (BERGSTRÖM, BORGSTRÖM, and ROTTENBERG, 1952).

obtained identical responses when he repeated these experiments and fed free acids and triglycerides respectively to rats and humans; *cf.* also SINGER, SPORN, and NECHELES (1953).

It is now clear that the route of absorption in these types of experiment is independent of the form in which the lipids are administered. The main reason for these discrepancies is therefore most likely to be the different rate of absorption from the intestine. It is well known that sizable amounts of free fatty acids administered to a fasting animal are more slowly absorbed than cor-

responding amounts of triglycerides, but the general condition of the animals, length of fast, temperature, etc., might also influence the results. Correspondingly, the rate at which the newly absorbed triglycerides enter the blood stream from the lymph is less in the first mentioned case. The result is that the amount of newly absorbed fat in the blood is kept lower during the absorption process. This is clearly indicated in the experiments shown in Fig. 7. The specific activity of the plasma triglyceride fatty acids shows a distinct peak during the absorption of triglycerides whereas the activity remained low in a corresponding experiment when labelled free fatty acids had been administered (BERGSTRÖM, BORGSTRÖM, and ROTTENBERG, 1952). As the concentration in the blood also depends on the rate at which the newly absorbed fat is removed into the different tissues, it is clear that the actual concentration of newly absorbed fat in the blood during the absorption cannot yield much information as to the rate or route of fat absorption. The value of chylomicron counts seems unsuitable for studies of this kind and has clearly led to erroneous interpretations of the absorption process (*cf.* also JEFFRIES, 1954).

In this connection it might also be mentioned that FRAZER (*loc. cit.*) has reported differences in the distribution of Sudan III when this dye was dissolved in free oleic acid or in olive oil fed to rats. Only in the latter case did the lymphatics become coloured. In the former case more dye was observed in the liver. No description of the methods nor any quantitative data were given.

AULD and NEEDHAM (1951) have fed triglyceride and oleic acid containing Sudan III to a human case of chylothorax. In the former case 20 per cent of the dye was recovered from the chylous fluid withdrawn from the thoracic cavity whereas almost none was recovered in the latter case.

These results can possibly partly be explained by the data published by MOLANDER (1949) on the absorption of carotene dissolved in corn oil or in the free fatty acids from corn oil. The amounts absorbed were in the former case 69 per cent but in the latter only 20·8 per cent, but the absolute amounts deposited in the liver were similar in both cases. The main reason for these differences might be that in the former case the micelles that also contain different glycerides have a better solubilizing power and thus carry more dye into the intestinal cells. Data with carotene have to be interpreted with caution due to the metabolic changes that take place already in the intestinal wall (*cf.* BERNHARD, 1953). This type of experiment is unsuitable for studying the route of fat absorption (*cf.* also VERZAR, 1948).

It can thus be summarized that all acceptable evidence available now indicates that *long chain* ($\geq C_{16}$) acids *whether saturated or unsaturated and whether fed as free acids or as esters are predominantly absorbed via the lymphatics mainly as triglycerides and to a small degree incorporated into phospholipids.*

2. *Medium and short chain* (C_{15} to C_2) *carboxylic acids*—It has been known for a long time that the short chain acids, such as acetic, propionic and butyric, are rapidly absorbed from the intestine and earlier work has also indicated that they are transported *via* the portal vessels as would be expected (HUGHES and WIMMER, 1935).

Some early work by RAPER (1913) in which he fed cocoa fat to dogs and analysed the lymph fat is of interest in this connection. As the mean molecular weight of the fatty acids of the lymph lipids was considerably higher than in the fat fed he suggested that the lower acids were absorbed through some other channel than the chyle.

The work with glycerol-labelled tributyrin and triacetin (KARNOVSKY and GIDEZ, 1951; BERNHARD *et al.*, 1952) further indicates that these compounds are rapidly and completely split in the intestine.

BLOOM, CHAIKOFF, and REINHARDT (1951) have made a systematic study of the route of absorption of saturated fatty acids of different chain lengths in rats with lymph fistulas. While the acids with 16 to 18 carbon atoms as mentioned were almost entirely transported *via* the lymphatics, acids with 14, 12 and 10 atoms were found in the lymph in amounts decreasing with the chain lengths; decanoic appeared only to an extent of 5 to 19 per cent of that absorbed. Only about 8 mgm of each acid was fed in 0·5 ml oil but KIYASU, BLOOM, and CHAIKOFF (1952) have later shown that the activity after hydrolysis was present in the blood as an acid. The ratio between the activity present in the portal vessels and in the inferior vena cava was considerably greater than one (1·3 to 9·7). BORGSTRÖM (1955) has extended these studies on the absorption of decanoic acid in the rat and analysed the distribution of the labelled material in the portal and inferior vena cava blood. About two-thirds of the activity was present as free decanoic acid and the remainder was incorporated in neutral fat and phospholipids. The concentration of free acid in the portal blood reached about 5 mgm per cent.

FERNANDES *et al.* (1953, 1954) (*cf.* preceding section, p. 377, and Table 1 on p. 381) have also studied the fate of short chain fatty acids when fed incorporated into mixed glycerides given to the child with the chylothorax. The results agree rather closely with the work with saturated acids on rats mentioned above (BLOOM *et al.*, 1951). When fats containing both short chain and long chain acids were fed the former appeared in the lipids of the chyle in progressively lesser extents the shorter the chain. When a synthetic glyceride was fed that contained 38 per cent caprylic acid (C_8) and for the rest mostly oleic acid and a little palmitic and stearic acid ("caprylic olive oil"), the lymph lipids contained only about 4 per cent caprylic acid (*cf.* Table 1).

They also point out that from these data it is clear that the hydrolysis of the absorbed fat must have been very extensive.

3. *Substituted fatty acids*—It was mentioned in section IV that α,α-dimethyl stearic acid did not become incorporated into glyceride ester bonds when present during hydrolysis of a triglyceride with pancreatic lipase *in vitro*.

The absorption of this acid with a [^{14}C] carboxyl group has been investigated by BERGSTRÖM, BORGSTRÖM, TRYDING, and WESTÖÖ (1954) on rats with lymph fistulas.

When administered in olive oil it was well absorbed. It was recovered in high yield in the lymph lipids incorporated into both glyceride and phospholipids in the usual proportions. When the isolated lymph triglycerides were

Table 1. Fatty acid composition of fat fed and of lipids recovered from lymph obtained from child with spontaneous chylothorax (FERNANDES, 1953)

Acid	*"Caprylic olive oil"*		*Sunflower oil*		*Rape oil Groundnut oil*		*Coconut oil*	
	Fat fed	*Lymph fat*	*Fat fed*	*Lymph fat*	*Fat fed*	*Lymph fat*	*Fat fed*	*Lymph fat*
Caprylic (C_8)	28	4	0	0			8	0
Capric (C_{10})	0	0	0	0			6	2
Lauric (C_{12})	0	0	0	< 1	0	< 1	46	33
Myristic (C_{14})	0	0	0	1	1	2	17	14
Palmitic (C_{16})	8	16	6	8	9	11	9	13
Stearic (C_{18})	1	5	4	5	2	4	3	4
Arachidic (C_{20})					1	1		
Behenic (C_{22})					1	1		
Unsaturated (total)	54	75	90	86	86	80	11	34
Linoleic			53	49				
Linolenic			2·1	2·2				
Erucic					17	18		
Iodine number	46	82	128	127	102	97	8	35
Total fat in lymph		2·1		2·9		3·2		3·0

hydrolyzed *in vitro* with rat bile pancreatic juice the labelled dimethylstearic acid was split off very much slower than the normal acids, resulting in a ten-fold concentration of this acid in the remaining glycerides.

These experiments thus demonstrate, as expected, that the enzymic mechanism through which the ester bonds are formed in the cell is not a simple reversal of the overall hydrolysis of pancreatic lipase. The formation of ester bonds in the intestinal cells presumably takes place *via* CoA-S-acyl compounds formed at the expense of energy rich phosphate bonds as indicated by the work of KORNBERG and PRICER (1953). A marked specificity was also observed in their work with an enzyme preparation from liver. No data are available on corresponding enzymes present in the intestinal mucosa cells.

f. Extent of total hydrolysis and fate of glycerol during fat absorption

1. *Experiments with labelled glycerol*—The extent of hydrolysis of triglycerides in this section is considered as the extent to which glycerol is set free during the absorption process.

Recent work in several laboratories with labelled glycerol has contributed what appears to be the first unequivocal data as to the extent of total hydrolysis of triglycerides during intestinal absorption.

It has been confirmed that free glycerol is very rapidly absorbed. About 80 per cent of the isotopic carbon was recovered in the expired carbon dioxide in the following 24 hr (DOERSCHUK, 1951b).

When triglycerides or free fatty acids were administered simultaneously with free labelled glycerol, the latter was only incorporated to a very small extent into the glycerides or phospholipids present in the intestinal wall (FAVARGER and COLLET, 1950a,b; FAVARGER, COLLET, and CHERBULIEZ, 1951; BUENSOD, FAVARGER, and COLLET, 1953) nor did it appear in the lipids recovered from a thoracic duct fistula in amounts corresponding to more than an incorporation into a few per cent of the glyceride molecules (BERNHARD, WAGNER, and RITZEL, 1952). More of the isotope can, however, be recovered from the liver glycogen and a small amount also in the liver lipids (DOERSCHUK, 1951b), especially when fasted animals are used (BERNHARD and WAGNER, 1952). Slightly more isotope was present in the phospholipid fraction (KARNOVSKY and GIDEZ, 1951) than in the glycerides.

Free glycerol is thus rapidly and completely absorbed *via* the portal system. It is only utilized to a very small extent for the synthesis of triglycerides or phospholipids in the intestinal contents or cells when free fatty acids are absorbed and transported from the intestine as triglycerides in the lymph. In this connection it might be mentioned that in the *in vitro* systems in which BORGSTRÖM (1953) has found that pancreatic lipase causes the formation of triglycerides from monoglycerides, no incorporation of added free labelled glycerol took place, i.e. only substances forming micelles in the system take part in the synthesis under these conditions. *In vivo* this factor, in combination with the very rapid absorption of free glycerol, might contribute to the observed phenomenon.

The aforementioned data thus suggest that fed free glycerol is not utilized to any larger extent as precursor in the formation of triglycerides or phospholipids and that therefore any glycerol set free in the hydrolysis of long chain triglycerides is not reutilized for triglyceride synthesis in the intestine.

The authors of the papers referred to below have interpreted their data under this assumption.

When tributyrin or triacetin containing labelled glycerol were fed essentially the same results were obtained (KARNOVSKY and GIDEZ, 1951; BERNHARD *et al.*, 1952) as when free glycerol had been fed, indicating that a rapid and complete hydrolysis of these compounds takes place before they leave the intestinal cells.

BERNHARD *et al.* (1952) fed glycerol-labelled triolein or fat labelled both in the glycerol and fatty acids. When the lipids were recovered in the lymph the labelled glycerol had been much more diluted than the fatty acid. From these data he could calculate that a minimum of 25 to 53 per cent of the glycerides had been totally hydrolyzed. Essentially similar results with an estimated 25 to 45 per cent total hydrolysis were obtained simultaneously by REISER, BRYSON, CARR, and KUIKEN (1952) with ^{14}C-glycerol-labelled triglycerides in which the acids contained conjugated double bonds. They further reached the conclusion that the remainder of the triglycerides was broken down to monoglycerides. Their conclusion as to the amounts of mono- and diglycerides formed are further discussed on p. 385. In preliminary communications, GIDEZ and KARNOVSKY (1952) have reached estimates of the glycerol set free which are in the same range. They compared the rate of elimination of labelled carbon dioxide after feeding free [^{14}C] glycerol or glycerol labelled tributyrin or triolein. In 2 hr they estimated that 55 to 90 per cent of the tributyrin and 35 to 55 per cent of the triolein had been totally split whereas the figures were 100 per cent and 40 to 100 per cent respectively after 4 hr. These figures are based on the assumption that only free glycerol is oxidized, but the possibility of exchange reactions between glycerides and glycerol is not excluded.

The fate of monoglycerides during fat absorption has been further studied by REISER and WILLIAMS (1953). After feeding monopalmitin labelled both in the acid and glycerol moiety analysis of the lymph lipids confirmed older work (FRANK, 1898) that no monoglycerides were present, but they found that most of the labelled acid and about a quarter of the glycerol was present in the lymph triglycerides.

They also fed labelled 1-palmitoxy-3-hydroxyacetone and found that part of this compound had been reduced since labelled glycerol was recovered from the lymph triglycerides. As with the labelled monoglyceride, however, three-quarters of the labelled dihydroxyacetone was not recovered in the lymph. They suggested that dihydroxyacetone might serve as the precursor of glycerol during normal fatty acid absorption.

Their data, however, does not support the conclusion that free dihydroxyacetone is a better precursor for glyceride glycerol than free glycerol. The published experiment only shows that the monopalmitoyl esters of these two

compounds are utilized to the same extent for triglyceride synthesis during the absorption process.

Presumably blood glucose is the precursor of the glycerol of the newly formed triglycerides in the intestinal cells as it has been shown to be for the glycerol of the milk triglycerides in the rat and goat (FRENCH and POPJAK, 1951; POPJAK, GLASCOCK, and FOLLEY, 1952).

2. *Redistribution of fatty acids during intestinal absorption*—In the preceding section the extent of hydrolysis has been considered from the point of view of the amount of glycerol set totaliy free during the absorption of triglycerides. Most data had been obtained by comparison of the fat administered and the lipids recovered from a lymph fistula, i.e. the sum of the processes taking place in the intestinal lumen and in the intestinal cells was studied.

The extent of hydrolysis can also be considered from the point of view of the fatty acids that during this period are set free from the glyceride ester bond for a longer or shorter period of time.

The study of this process is, however, complicated by the fact that already in the intestinal content there is a rapid formation of glyceride ester bond, i.e. under the conditions of rapid overall hydrolysis the reversed reaction is not negligible (*cf.* sections IV*d* and V*b*). However, this reaction, that proceeds in the intestinal content, does not concern free glycerol but only the different glycerides. Positions 1 and 3, i.e. the two primary hydroxyls in the glycerol molecules are thus rapidly equilibrated with the free long chain fatty acids present. The ester bond at C_2 is, however, very slowly exchanged. As mentioned earlier at least four-fifths of the monoglyceride that is formed under the influence of pancreatic lipase is the 2-monoglyceride. How far these processes have proceeded under *in vivo* conditions in the intestinal content is not quite clear and certainly varies under different conditions and in different animals, *cf.* section V*a*. The exchange is presumably continued in the cell when a net synthesis predominates and where the mechanism of ester bond formation is different from that catalyzed by the pancreatic lipase as evidenced by the results with [^{14}C] α,α-dimethylstearic acid. This acid is not incorporated into glyceride ester bonds with pancreatic lipase *in vitro* but it appears in the triglycerides and phospholipids of the lymph (BERGSTRÖM *et al.*, 1954) (*cf.* pp. 369 and 380).

The following experiment by BORGSTRÖM (1952c) indicates that a practically complete exchange of a glyceride fatty acid can take place when triglycerides are absorbed. Under similar conditions experiments with glycerol labelled triglycerides indicate a total hydrolysis of only one-third to one-half of the fat absorbed, i.e. one-third to one-half of the labelled glycerol in relation to the fatty acids of the fat recovered in the lymph has disappeared.

Three groups of rats with lymph fistula received the same mixture of corn oil containing about 4 per cent of cholesterol. [^{14}C] Stearic acid was present in corresponding amounts in each mixture but in different form (*cf.* Fig. 8). In mixture (1) the labelled acid was incorporated into glyceride ester bonds by transesterification; in mixture (2) the acid was dissolved in free form and in

(3) it was present esterified with cholesterol. When the distribution of the labelled acid in the lymph lipids was investigated it was found that the distribution was approximately the same in each group, i.e. roughly about 88 per cent in the neutral fat, 10 per cent in the phospholipids and the remainder as cholesterol esters. The specific activity of the fatty acids from these different lipids was also found to be roughly identical between the three groups.

This indicates, for example, that when a small amount of free labelled acid is given dissolved in triglyceride this acid is diluted to the same extent as when the same acid was given incorporated into glyceride ester bond at the feeding.

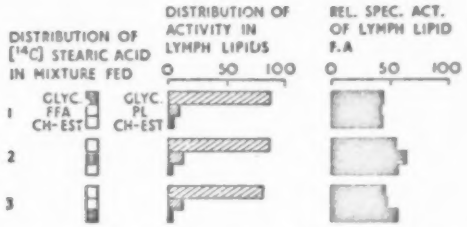

Fig. 8. Distribution and specific activity of fatty acids of glycerides, phospholipids and cholesterol esters in the lymph after administration of labelled stearic acid (1) incorporated into corn oil, (2) dissolved as free acid in corn oil and (3) esterified with cholesterol. (Borgström, 1952c.)

The results of group 3 furthermore indicate that the cholesterol esters must have been totally hydrolyzed during the process of absorption as the labelled acid is distributed in the same way whether fed as cholesterol ester or in free form. Total hydrolysis in this case again does not signify that all the cholesterol ester is totally hydrolyzed at one time but the possibility of an exchange at the active centre of the enzyme is also possible.

Reiser *et al.* (1952) have made a different type of experiment. They fed a mixture of an unlabelled saturated triglyceride (bayberry tallow) and a triglyceride synthesized from [^{14}C] glycerol and conjugated linoleic acid. The lymph fat was then subjected to a crude fractionation with alcohol-acetone at 5° in which the triglycerides containing two or three saturated acids are relatively insoluble and the content of labelled glycerol respectively of conjugated linoleic acid was determined. When equal amounts of the two fats had been fed, they estimated that 40 per cent of the triglyceride had been totally hydrolysed as calculated from the dilution of the labelled glycerol in the lymph lipids.

When the "saturated" and "unsaturated" glyceride fractions were analysed they found both the content of linoleic acid and also that of labelled glycerol to be lower in the "saturated" fraction. This indicates that the exchange in this case had not been complete but that presumably part of the 2-monoglycerides had not been totally exchanged. Reiser *et al.* estimated that the glyceride molecules that had not been totally split had been mainly degraded

to monoglyceride, i.e. 80 per cent of the glyceride ester bonds had been hydrolysed during the absorption process when a total hydrolysis of 40 per cent of the triglyceride molecules had taken place. The occurrence of an exchange of free fatty acids and glyceride fatty acids in the lumen of the small intestine *cf* section Vb makes the calculations of REISER *et. al.* (1952) invalid as to the species of glycerides absorbed.

The work reviewed in this section has to be extended in various directions to make more exact calculations possible. It is, however, clear that even if total hydrolysis in the sense of liberation of glycerol is limited in long chain glycerides, the remaining part of the triglyceride is very extensively degraded. Differences must again be expected to be found between different fats and different animals.

VI. INTESTINAL ABSORPTION OF HYDROCARBONS, ETC.

The results of studies on the absorption of hydrocarbons have played an important role in the development of theories on the intestinal absorption of triglycerides.

Earlier investigators were of the opinion that hydrocarbons such as liquid paraffin or vaseline could not be absorbed (HENRIQUES and HANSEN, 1900; BLOOR, 1913). However, BRADLEY and GASSER (1911) claimed to have obtained a sizable percentage of hydrocarbons from the lymph lipids after feeding a mixture of olive oil and petroleum oil to dogs. The data in BLOOR's (1913) paper also indicate that some absorption did take place.

The first real evidence that the hydrocarbons in liquid paraffin are absorbed when administered with the food to rats and pigs was supplied by CHANNON and his collaborators (CHANNON and COLLINSON, 1928, 1929; CHANNON and DEVINE, 1934; EL MAHDI and CHANNON, 1935) and they also found that the absorbed hydrocarbons had apparently been metabolized in the body. A comprehensive study on intestinal absorption of different paraffin oils in rabbits, guinea pigs and rats has been published by STRYKER (1941).

The issue was definitely settled by STETTEN (1943) who fed rats with deuterium labelled hexadecane mixed into the fat of the diet. He confirmed that it was absorbed to a considerable extent and further isolated deuterated fatty acids from the body of the animals, proving that the hydrocarbons had been metabolized, presumably by ω-oxidation.

These findings are, in principle, confirmation of THIERFELDER and KLENK's (1924) demonstration that phenyl substituted hydrocarbons are ω-(methyl)- and β-oxidized in the animal body.

It was thus definitely established in 1943 that hydrocarbons could be absorbed when supplied with the diet and that the absorbed hydrocarbons were extensively metabolized in the body.

FRAZER, SCHULMAN, and STEWART (1944) reported that liquid paraffin could be absorbed by fasting rats provided it was introduced directly into the upper small intestine as an emulsion with particles less than 0·5μ in diameter.

LUNDBACK and MAALØE (1946) were unable to reproduce these results. That a considerable part of paraffin oil is absorbed when fed to rats was also shown by MOLANDER (1949). That paraffin is absorbed fairly readily in the rat when administered dissolved in glycerides has again been confirmed (FRAZER, FRENCH, SAMMONS, and DANIEL, 1951).

A systematic study of the absorption of deuterated straight chain hydrocarbons has been made by BERNHARD *et al.* (1952), BERNHARD (1953) and by GLOOR (1953). They followed the absorption and metabolism of C_8 to C_{18} hydrocarbons and also analysed the hydrocarbons present in the lymph lipids. When the hydrocarbon was administered in a 20 per cent solution in olive oil, they recovered about 20 per cent of the hydrocarbon in the lymph. The presence of labelled fatty acids could not be demonstrated in the lymph, but a dehydrogenation-hydrogenation was indicated to have taken place during the passage through the intestinal mucosa since a considerable reduction of the isotope was demonstrated when 1,2-deuterated hydrocarbons had been fed. A further extensive metabolism with ω- and β-oxidation was also shown to take place in the body (*cf* also p. 386).

As to the absorption of hydrocarbons it is clear that a considerable absorption can take place. The extent is dependent on the properties of the hydrocarbon, the way in which it is supplied (dissolved in triglycerides, etc.). Presumably no absorption takes place in the absence of bile as is known to be the case for cholesterol. There are indications that even saturated hydrocarbons are already attacked metabolically to some extent during the passage through the intestinal cells.

A neutral, lipid soluble compound that is extensively metabolized during passage through the intestinal cells is cetyl alcohol. It was known by STETTEN and SCHOENHEIMER (1940) that it was well absorbed by rats and that part of it could be recovered as palmitic acid from the body.

Recently BLOMSTRAND and RUMPF (1954) have extended these studies with cetyl alcohol-1-^{14}C, which was fed to rats with lymph fistulas. More than 50 per cent of the absorbed activity was recovered from the lymph lipids. Only about one-tenth was present as unchanged free cetyl alcohol whereas the remainder had been oxidized to palmitic acid which was incorporated into lymph triglycerides and phospholipids in the proportions characteristic of this acid.

This oxidation is thus already mostly taking place in the intestinal cells and again gives an indication of the intense metabolic activity in these cells.

ADDENDUM

The views on the structure of the intestinal mucosa and especially of the "cannals of BAKER" of the inner border of the cells have played a prominent part in the discussions on the fat absorption mechanism (FRAZER *et al.*, *loc. cit.*).

As new experimental evidence has accumulated recently we have asked two experts in the field to write a short summary on the subject.

VII. Structure of Intestinal Mucosa

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Since the first observations of a striated appearance of the so-called striated border of the intestinal epithelium cells two interpretations have been discussed. According to Funke (1855) and Kölliker (1856) the striations are due to minute cylindrical canals penetrating a thick cell membrane. Brettauer and Steinach (1857), on the other hand, considered the striations as due to minute rod-shaped structures.

According to the light microscopic analysis by J. R. Baker (1942) the border of the cells consists of a continuous layer perforated by spindle-shaped canals.

Electron microscopic studies (Granger and R. F. Baker, 1949, 1950) on sections have revealed that the striated border consists of 80 mμ thick and 620 mμ long cylindrical processes oriented perpendicularly to the cell surface. Similar observations were made by Dalton *et al.* (1950). The resolution of these electron micrographs was not sufficient to make the cell border stand out as a separate well-defined structure and no definite conclusions could be drawn as regards the relations of these processes to the cell membrane at, for instance, their base. The authors concluded that the processes extended through the cell membrane from an opaque region in the interior of the cells.

In electron micrographs at higher resolution of ultra-thin sections (Sjöstrand and Zetterqvist, 1954) it has been possible to observe the cell membrane and thus to clarify further the organization of the striated border. The cell membrane at the free surface of the intestinal epithelium cells in the small intestines of the mouse appears as a double line The total thickness of the cell membrane is about 120 Å, the thickness of the dark lines about 45 Å and the width of the space in between these lines about 30 Å. It might tentatively be proposed that this double appearance presumably is due to the cell membrane being composed of two protein layers separated by a lipid layer as suggested for the similar structure appearing in the mitochondria (Sjöstrand, 1953; Sjöstrand and Rhodin, 1953, *cf* Figs. 9–12).

In these pictures it can be demonstrated that the cylindrical processes represent finger-like extensions of the cell membrane and that they do not penetrate the cell surface to reach the interior of the cell. The processes are covered by the cell membrane all over their surface, no pores or discontinuities being observed. The interior of the processes consists of a cytoplasm containing a vaguely defined longitudinal fibrillar structure of minute dimensions. At the base the cytoplasm of the processes seems to extend into the cell forming "roots" characterized by a somewhat higher opacity than the surrounding cytoplasm of the cell body and with diffuse outlines. On thicker sections these extensions form an opaque continuous layer in the apical part of the cells, a fact that explains the observations made by Granger and R. F. Baker.

From a functional point of view it is important to consider that the cell

Fig. 9. Slightly oblique section along the striated border of the intestinal epithelium of the mouse jejunum. Magnification × 71000. (SJÖSTRAND and ZETTERQVIST, 1954.)

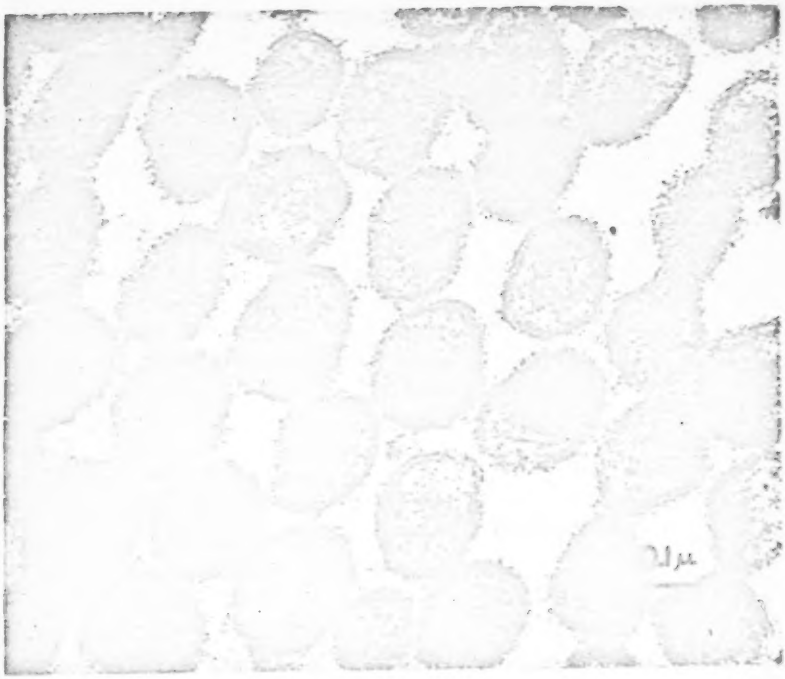

Fig. 10. Transversal section through the striated border of the intestinal epithelium of the mouse jejunum. The transversally cut processes are bounded by a double-edged membrane. Magnification × 137000. (SJÖSTRAND and ZETTERQVIST, 1954.)

Fig. 11. Section through the basal part of the striated border of the intestinal epithelium of the mouse jejunum. The cell membrane forms a continuous boundary on the processes as well as between their bases. Magnification × 92000. (SJÖSTRAND and ZETTERQVIST, 1954.)

Fig. 12. Longitudinal section through the tips of the striated border processes, jejunum, mouse. At the tips the processes are bounded by the double-edged cell membrane. Magnification × 97000. (SJÖSTRAND and ZETTERQVIST, 1954.)

surface is continuously coated by a very well-defined cell membrane and that no pores or holes in the membrane are present.

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11

METABOLISM OF THE STEROID HORMONES

Leo T. Samuels

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METABOLISM OF THE STEROID HORMONES

Leo T. Samuels

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1. Introduction

Before one may discuss the metabolism of the steroid hormones it is necessary to know what compounds belong in this classification. This involves the establishment of both chemical and physiological criteria. Although the term "steroid hormone" has been applied to substances secreted by the interstitial cells of the testis, the ovarian follicle, the corpus luteum, the adrenal cortex, and the placenta, it is only in the last five years that any compound has been clearly established as such, and in the case of the ovarian follicle the evidence is not yet conclusive.

To understand the reasons for this, one must consider what is necessary to establish a compound as belonging to this class. Chemically, the compound must be demonstrated to contain the perhydrocyclopenta (a) phenanthrene nucleus characteristic of the sterols. Physiologically, the compound must be demonstrated to be formed in an endocrine gland, secreted into the blood stream by the gland, and to have a physiological action which is lacking when symptoms of glandular deficiency develop. By the end of 1937 compounds had been found in each of the organs mentioned which met all of the criteria except the evidence that they were actually secreted into the blood. This required the development of micro techniques which have only recently become available.

Since a consideration of steroid hormones and their metabolism involves the use of names to indicate structure, a brief description of structural nomenclature will help in orientation. In Fig. 1 the perhydrocyclopenta (a) phenanthrene nucleus with the side chain as in cholesterol (cholestane) is given with the carbons numbered. The numbers in heavy type indicate the positions most commonly involved in biological reactions. The angular methyl groups 18 and 19, attached to carbons 13 and 10 respectively, lie on the same side of the plane of the molecule and are used as reference groups for stereochemical designation. All groups *cis* to the angular methyl groups are designated "β," while those *trans* to the methyl groups are "α."

The relations of rings B : C and C : D are constant in the steroid hormones and their products, but the union of A : B may occur with the H atom at

carbon 5 in either α or β position. This gives rise to two basic types for each carbon skeleton, each having its own root name.

During the development of steroid chemistry names were given before the structural relationships were known. Recently a conference was held to attempt a systematization of steroid nomenclature (Ciba Foundation Conference, 1951). The recommendations of this conference have been followed in this review with certain exceptions. When an epimer of a compound having an accepted trivial name is referred to, the prefix epi- is used, e.g. androsterone and epiandrosterone. The established designation "etiocholane" has been used for the nucleus resulting from removal of the side chain instead of the recommended "testane" because the latter implies an association with the testis.

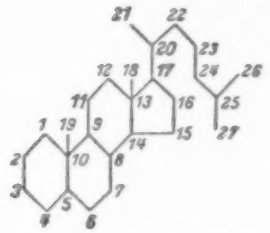


Fig. 1. Chelestane, showing numbering of carbon atoms. Bold face type indicates position frequently involved in the biochemical reactions.

Lastly the designations for unsaturated compounds have been written with the position of double bonds indicated before the name, e.g. 5-pregnen-3β-ol-20-one, instead of the recommended preg-5-en-3β-ol-20-one which is hard to pronounce.

In 1929 Doisy *et al.* and Butenandt independently isolated estrone (I; Fig. 2) from the urine of pregnant women. This compound produced estrus in ovariectomized animals and caused physiological changes in ovariectomized women simulating the first half of the menstrual cycle. It was therefore called an estrogen. In 1930 Marrian isolated estriol (II). By 1933 Butenandt *et al.* had established the perhydrocyclopenta (a) phenanthrene nucleus in estrone and estriol. In 1935 MacCorquodale *et al.* isolated estradiol (III) from an extract of hog ovaries, a compound which had been obtained previously from estrone by reduction of the ketone group (Schwenk and Hildebrandt, 1933). They found that estradiol accounted for most of the estrogenic activity of the ovarian extract; the balance appeared to be due to a compound which resembled estrone chemically and physiologically but which was not obtained in crystalline form. The final criterion, that of secretion, has not been clearly proved for either of these steroids, however.

Urine did not prove to be a source of the lipid hormone of the corpus luteum which caused the progestational changes in the uterus, but in 1934 four different laboratories (Allen and Wintersteiner, 1934; Butenandt and Westphal, 1934; Slotta *et al.*, 1934a; Hartmann and Wettstein, 1934) succeeded in

isolating the highly active compound progesterone (IV) from hog corpora lutea. Very shortly thereafter, the structure of progesterone was established by SLOTTA *et al.* (1934b). The steroid was isolated from adrenal cortex extracts

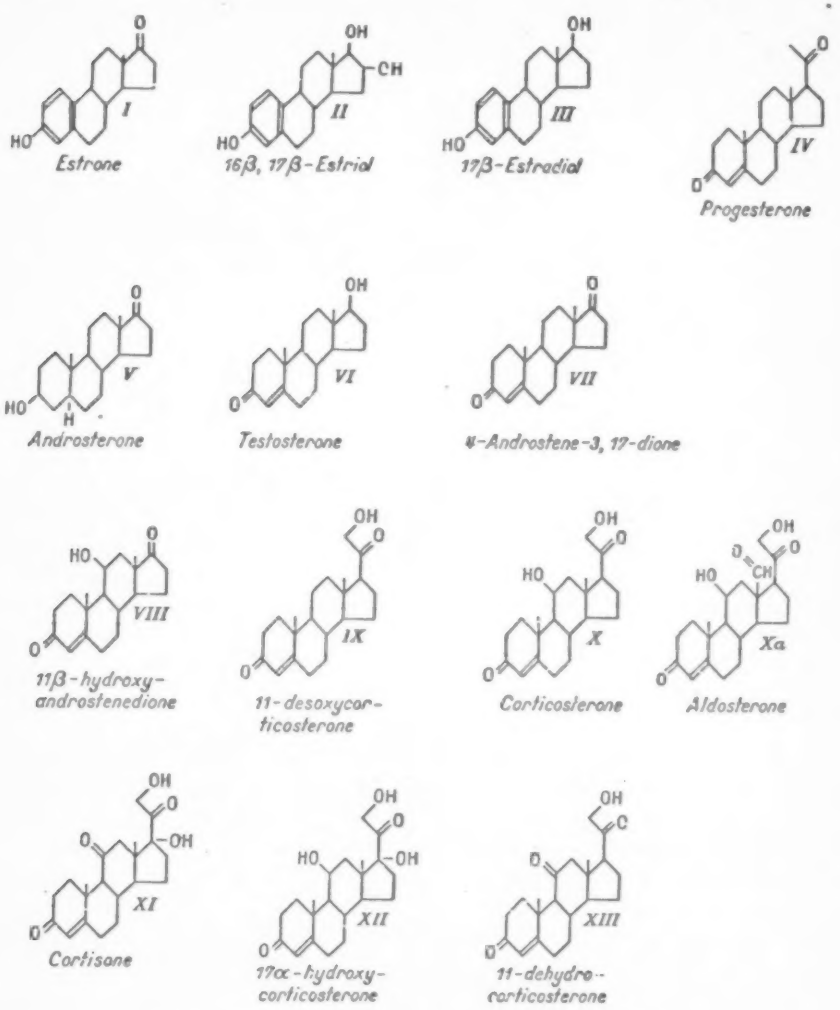


Fig. 2. Steroid hormones and historically related compounds.

in 1938 by BEALL and REICHSTEIN. Its isolation from placentae was not achieved, however, until 1952 when SALHANICK *et al.*, PEARLMAN and CERCEO, and DICZFALUSY independently announced its separation.

BUTT *et al.* (1951b) reported that a compound which moved on their chromatographic column like progesterone and which gave an ultraviolet absorption

peak at 240 mμ, similar to that for an α,β unsaturated ketone, was found in 2 out of 5 samples of human placental blood. It was not found in the general circulation. PEARLMAN and THOMAS (1953) have confirmed these observations. In 1952 EDGAR reported that paper chromatography of an extract of ovarian vein blood of sheep yielded a spot which moved in the particular solvent system like progesterone, was an α,β unsaturated ketone, and did not contain any alcohol groups. No such spot was found in peripheral venous blood. While the evidence is not conclusive, it seems highly probable that progesterone is secreted by the ovary of the sheep. Since it has been isolated from a number of animal sources, including human placenta, and since no other compound having a similar degree of progestational action has been found in natural products it seems highly likely that progesterone is the progestational hormone secreted by the corpus luteum and placenta in most, if not all, species.

In 1931 BUTENANDT isolated crystalline androsterone (V) from human urine, and RUZICKA *et al.* (1934) proved its steroid character by synthesis from cholesterol. The androgenic material in extracts of bull testes showed different chemical characteristics, however, and in 1935 DAVID *et al.* obtained pure testosterone (VI) from this source. In the same year both BUTENANDT and HANISCH (1935), and RUZICKA (1935) succeeded in synthesizing this compound from cholesterol via dehydroepiandrosterone (XXI).

Conclusive evidence of the nature of the secretory products of the testis has only recently been found, however. WEST *et al.* in 1952 reported the isolation of testosterone and 4-androstene-3, 17-dione (VII) from the spermatic vein blood of dogs. They demonstrated that the amounts were much higher than in the general circulation. In the same year SAVARD and co-workers (1952) introduced C^{14}-acetate into fluid perfused through a human testis, and obtained testosterone and androstenedione labelled with the isotope. BRADY (1951) had already reported that slices of rabbit, dog, pig and human testis incubated with C^{14}-acetate yielded testosterone-C^{14} as determined by migration in paper chromatography. It seems fairly well established, therefore, that 4-androstene-3,17-dione and testosterone are both testicular hormones.

The hormones of the adrenal cortex which control the physiological functions peculiarly associated with that tissue were the last to be isolated but the first to be established as hormones. A wide range of compounds, all demonstrated to be steroid derivatives, were isolated from adrenal extracts by REICHSTEIN, by KENDALL and MASON and by WINTERSTEINER and PFIFFNER during the years 1936–38 (REICHSTEIN and SHOPPEE, 1945). Among these were androgenic compounds, estrone, progesterone, and several steroids with no obvious physiological activity. But in addition, there were desoxycorticosterone (IX) which showed high activity on electrolyte balance, corticosterone (X) which had both metabolic and electrolyte effects, and cortisone (11-dehydro-17α-hydroxycorticosterone, XI) and 17α-hydroxycorticosterone (XII) which were most active metabolically.

VOGT (1943) showed that adrenal vein blood contained a lipid substance which would protect adrenalectomized rats against cold. The amounts were

much higher than in peripheral blood and rose on the injection of adrenocorticotropic hormone of the pituitary gland (ACTH).

HECHTER *et al.* (1949) perfused cow adrenals and were able to demonstrate increased metabolic effect with lipid extracts of the perfusate, particularly when ACTH was added. They were able to isolate a number of hormonally active steroids from this material. In 1950 NELSON *et al.* reported the isolation of 17α-hydroxycorticosterone (compound F) from the adrenal vein blood of dogs injected with ACTH. Shortly thereafter, REICH *et al.* (1950) succeeded in isolating, in addition, small amounts of corticosterone from this source. No cortisone could be identified.

BUSH (1953a) also studied the steroids in the adrenal vein blood of different species and found a marked variation. In most species 17α-hydroxycorticosterone was the major hormonally active component, but in the rat corticosterone was present in greatest amount. He also identified small amounts of 11-dehydrocorticosterone (XIII) and, occasionally, cortisone in the adrenal venous blood of some species. The relative concentrations of the different steroids in a given species were generally maintained during stimulation with exogenous ACTH. In summary, then, it may be concluded that 17α-hydroxycorticosterone and corticosterone have been established as hormones of the adrenal cortex, their relative importance varying among different species, and that cortisone and 11-dehydrocorticosterone may be secreted in small amounts.

It was observed by PFIFFNER *et al.* (1935) that a fraction which they could only obtain from adrenal extracts in amorphous form was much more active in maintaining the life of adrenalectomized dogs than 11-desoxycorticosterone, the most active crystalline compound then known. Recently GRUNDY *et al.* (1952), using paper chromatographic methods, were able to demonstrate the presence of a substance in adrenal extracts more polar than desoxycorticosterone which had very high electrolyte activity. This same substance was demonstrated by SIMPSON *et al.* (1952) to be present in adrenal vein blood but could not be found by their technique in peripheral blood. This also, then, appears to be a hormone secreted by the adrenal cortex. It has been obtained in crystalline form by SIMPSON *et al.* (1953) and by MATTOX *et al.* (1953), and its structure has recently been established by SIMPSON *et al.* (1954), who have given it the name aldosterone (Xa). This appears to be the major hormone of the adrenal cortex affecting electrolyte metabolism.

In 1951 GASSNER *et al.* reported the preparation of a fraction from bovine adrenal vein blood which gave the reaction of a 17-ketosteroid and was androgenic. The fraction was not measurable in the general circulation. In the adrenal vein blood of rats and some cats BUSH (1953a) found 4-androsten-11β-ol-3,17-dione (11β-hydroxyandrostenedione) (VIII). SALAMON and DOBRINER (1953) isolated this compound from the urine of a patient who had been treated with large amounts of ACTH, and ROMANOFF *et al.* (1953a) isolated it from human adrenal vein blood. Since α,β unsaturated ketonic steroids are not usually produced outside the endocrine system it seems probable that

4-androsten-11β-ol-3,17-dione is also secreted by the human adrenal. Such substances, then, also appear to be secretory products of the adrenal glands.

On the basis of the evidence reported, therefore, we may consider that we are talking about reactions related to the following compounds when we discuss the metabolism of the steroid hormones: estrone, estriol, estradiol, progesterone, testosterone, 4-androstene-3,17-dione, corticosterone, 17α-hydroxycorticosterone, 17α-hydroxy-18-aldocorticosterone (aldosterone), and certain 11-oxy-17-ketosteroids.

As with all reviews in an active field, this will probably be out of date by the time it appears in print. Undoubtedly some of the uncertainties cited above will have been resolved, and other compounds will have been added to the list of steroids secreted by the endocrine glands.

II. The Biosynthesis of the Steroid Hormones

Although there are many gaps, the general pattern of the biosynthesis of the steroid hormones is emerging. The outstanding feature of this pattern is its similarity in the different tissues. A common basic sequence appears to be followed, the differences being only in the later stages of formation where the relative activity of an enzyme system determines the ultimate product of the gland. The similarity in the early steps of synthesis may be a reflection of the common origin of these steroid-forming tissues from the region of the genital ridge.

A. Acetate as a precursor of the steroids

The basic role of the two carbon fragment in the formation of the steroid nucleus is brought out by the numerous studies on the synthesis of cholesterol (XIV), to which the laboratories of Bloch, Gurin, and Chaikoff have been particularly important contributors. That the same is true for the steroid hormones is indicated by the demonstrations that acetate can be incorporated into the adrenal hormones, the testicular hormones and the estrogens. Whether the incorporation in the hormones is *via* cholesterol has not as yet been clearly determined.

While the extensive literature on the synthesis of cholesterol will not be reviewed here, it is pertinent in view of the possible intermediate role played by this sterol to notice the extent to which the acetate group appears directly involved in its formation.

The work of Bloch's group on liver cholesterol has been the most exhaustive on mechanism of synthesis. These workers (Little and Bloch, 1950; Bloch, 1951) incubated liver slices with 2-C^{13},1-C^{14}-acetate and determined the ratio of methyl to carboxyl carbons in the entire molecule, the side chain and the nucleus of the cholesterol which was isolated. The respective ratios were 1·27, 1·08, and 1·48. The distribution of methyl and carboxyl carbons in the cholesterol molecule which would most nearly fit these, assuming that all carbons came from acetate, would be respectively 15/12 (1·25), 10/9 (1·11), and 5/3 (1·67). The ratio of 1·25 in the entire molecule was confirmed by Brady and Gurin (1951).

LITTLE and BLOCH and WUERCH *et al.* (1952) were able to determine the type of isotope in each of the carbons of the side chain and in the angular methyl groups of the nucleus. Carbons 21, 22, 24, 26 and 27 of the side chain, as well as the two angular methyl carbons and carbon 17 of the nucleus, were from the methyl carbons of acetate; carbons 10, 20, 23 and 25 were from carboxyl. This would fit with the concept of an alternation in the main sequence with all the angular groups being derived from methyl. Such a scheme would fit the

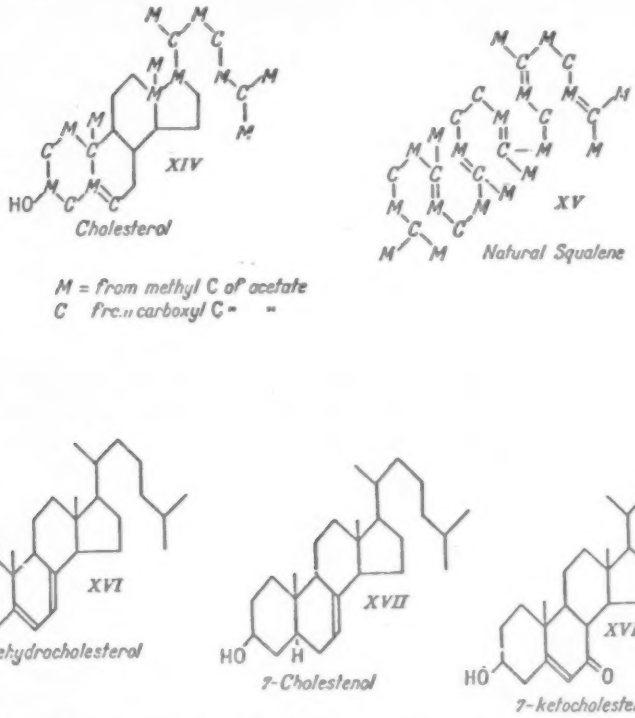


Fig. 3. Compounds possibly involved in synthesis of steroids from acetate.

ratios postulated. This work also indicated that all the carbons of cholesterol originated from acetate since the concentration of isotope in a specific carbon was the same as that calculated from the concentration of the isotope in the whole molecule and the number of carbons postulated as derived from the carbon of acetate which was so labelled.

The sequential relationships in the ring structures themselves have been difficult to establish. Recently, however, CORNFORTH *et al.* (1953 a,b,c), using a new degradation, were able to determine the origin of the carbons in ring A and carbon 19. WOODWARD and BLOCH (1953) determined the source of carbon 13. The results fit with the hypothesis of distribution already cited. Fig. 3 gives the source of the carbons which have been identified.

The regular pattern of distribution of acetate carbons led BLOCH (1951) to consider that cholesterol might be formed from the isoprenoid hydrocarbon squalene (XV) as originally postulated by CHANNON (1926). LANGDON and BLOCH (1952) tested this hypothesis experimentally. They first fed squalene from shark livers to rats, together with isotopic acetate, and isolated isotopic squalene from the livers, indicating mammalian synthesis. They then showed that this isotopic squalene was used more efficiently for cholesterol synthesis than was any other precursor thus far tried. Synthetic squalene, a different isomer, was not effective (LANGDON and BLOCH, 1953a).

The specific relation of the natural isomer of squalene to cholesterol synthesis in the liver has received support from the experiments of CHAIKOFF's group. SREBE (1951) found that the simultaneous feeding of natural squalene decreased the incorporation of acetate into cholesterol in the rat liver. LANGDON and BLOCH (1953b) and TOMKINS *et al.* (1953) confirmed this and showed that the synthetic isomer had no effect. If squalene itself is not the direct precursor of cholesterol, some closely related compound must play such a role.

LANGDON and BLOCH (1953c) also tried out other possible precursors of cholesterol in the competitive feeding test. 7-Dehydrocholesterol (XVI), found in skin (GLOVER *et al.* 1952), and 7-cholestenol (XVII), found by FIESER (1951) associated with cholesterol in most tissues, acted as marked inhibitors of 1-C^{14}-acetate incorporation in hepatic cholesterol. At the same time, as with natural squalene, total cholesterol was increased. When LANGDON and BLOCH (1953c) fed squalene, 7-cholestenol accumulated in the liver. They think, therefore, that 7-cholestenol may be an intermediate between squalene and cholesterol.

WEST *et al.* (1952) found, in addition to the testicular hormones, a relatively high concentration of 7-ketocholesterol (5-cholesten-3$^{\beta}$ ol-7-one, XVIII) in spermatic vein blood. Perhaps this represents another intermediate in steroid synthesis.

The use of isotopes has also made it possible to establish acetate as a precursor of the adrenocortical hormones, the androgens and the estrogens. The conversion has been demonstrated in the adrenals and testes and indicated in the ovaries. The formation of progesterone from acetate in the corpus luteum has not yet been reported but probably will be in the near future.

ZAFFARONI *et al.* (1951) perfused bovine adrenals with 1-C^{14}-acetate and ACTH and found the isotope incorporated in all the adrenal steroids isolated. HAINES *et al.* (1951) incubated adrenal slices with labelled acetate and ACTH and found radioactivity in 17-hydroxycorticosterone. After incubating testis slices with isotopic acetate, BRADY (1951) added testosterone, extracted the mixture, and chromatographed the extracts on paper. Radioactivity was present in the testosterone and cholesterol spots. SAVARD and co-workers (1952) perfused human testes with solutions containing radioacetate and obtained radioactivity in testosterone and 4-androstene-3.17-dione isolated from the perfusate after addition of unmarked carrier steroids.

WERTHESSEN and co-workers (1953) perfused sow ovaries with radioacetate. They found small amounts of radioactivity in recrystallized estrone and

estradiol after the addition of carrier material to the extracts. The activity was so low, however, that the danger of slight contamination left the conversion open to some question. HEARD *et al.* (1954) isolated estrone, equilin and equilenin from the urine of a pregnant mare after injection of 1-C^{14}-acetate. All three estrogens were radioactive. This appeared to be limited to the alicyclic carbons, indicating a different source for the aromatic rings.

Thus the evidence is good that the two carbon group is the basic unit in the formation of all those steroids, hormonal and non-hormonal, where this process has been studied. Whether the arrangement of acetate carbons in the steroid hormone molecules is the same as in cholesterol remains to be determined. Certain substances appear to be intermediates, or closely related thereto, in the synthesis of cholesterol, but their relation to steroid hormone synthesis has not yet been studied.

B. Cholesterol as a precursor of the steroid hormones

The concept that cholesterol is the precursor of the steroid hormones arose as soon as their steroid character was established. The hypothesis is supported by evidence that all of the endocrine tissues producing these hormones which have been investigated synthesize cholesterol from acetate; that the concentration of cholesterol undergoes changes associated with hormone secretion; and finally, that isotopic cholesterol can be converted into steroid hormones by perfusion through the adrenal cortex. Whether cholesterol is an essential intermediate in steroid hormone synthesis, however, has not been established.

SRERE, CHAIKOFF, and DAUBEN (1948) reported that acetate is incorporated into cholesterol by adrenal slices. BRADY (1951) found isotope in cholesterol isolated from testis slices after incubating with 1-C^{14}-acetate, and WERTHESSEN *et al.* (1953) found isotope in cholesterol of ovaries perfused with isotopic acetate. POPJAK and TIETZ (1953) obtained as active incorporation of acetate into cholesterol by the ovarian membranes (theca interna and granulosa) as that obtained with liver slices. HEARD *et al.* (1954) found incorporation of isotope into the cholesterol of the foetal placenta after injection of 1-C^{14}-acetate into a pregnant mare. It seems, therefore, that the synthesis of cholesterol from acetate occurs readily in the tissues where steroid hormones are formed.

Changes in the concentration of cholesterol in the adrenal cortex and ovaries appear to be related to alterations in the rate of secretion of the steroid hormones by these tissues. The most striking change is in the adrenal cortex, probably because hormone secretion from this tissue undergoes the most rapid fluctuation. LAIGNEL-LAVASTINE in 1918 called attention to the fall in adrenal cholesterol when patients died after prolonged motor activity. He noted that individuals dying of the same conditions but without any sustained period of activity showed less reduction. It required another 26 years before the significance of this observation was appreciated. In 1944 SAYERS *et al.* confirmed this change; in addition, they demonstrated that the cholesterol level of the

rat adrenal fell after injection of ACTH and that the fall was proportionate to the dose. LONG (1947), therefore, looked upon this as the precursor of the adrenal steroids released under the influence of the tropic hormone. VESTLING and LATA (1951) incubated adrenal homogenates in the presence of added cytochrome c and diphosphopyridine nucleotide, and found that cholesterol decreased while a compound which moved in a paper chromatographic system like 17-hydroxycorticosterone increased. This result also fits with the theory of cholesterol as a precursor but does not prove that cholesterol was the source of the hormone formed.

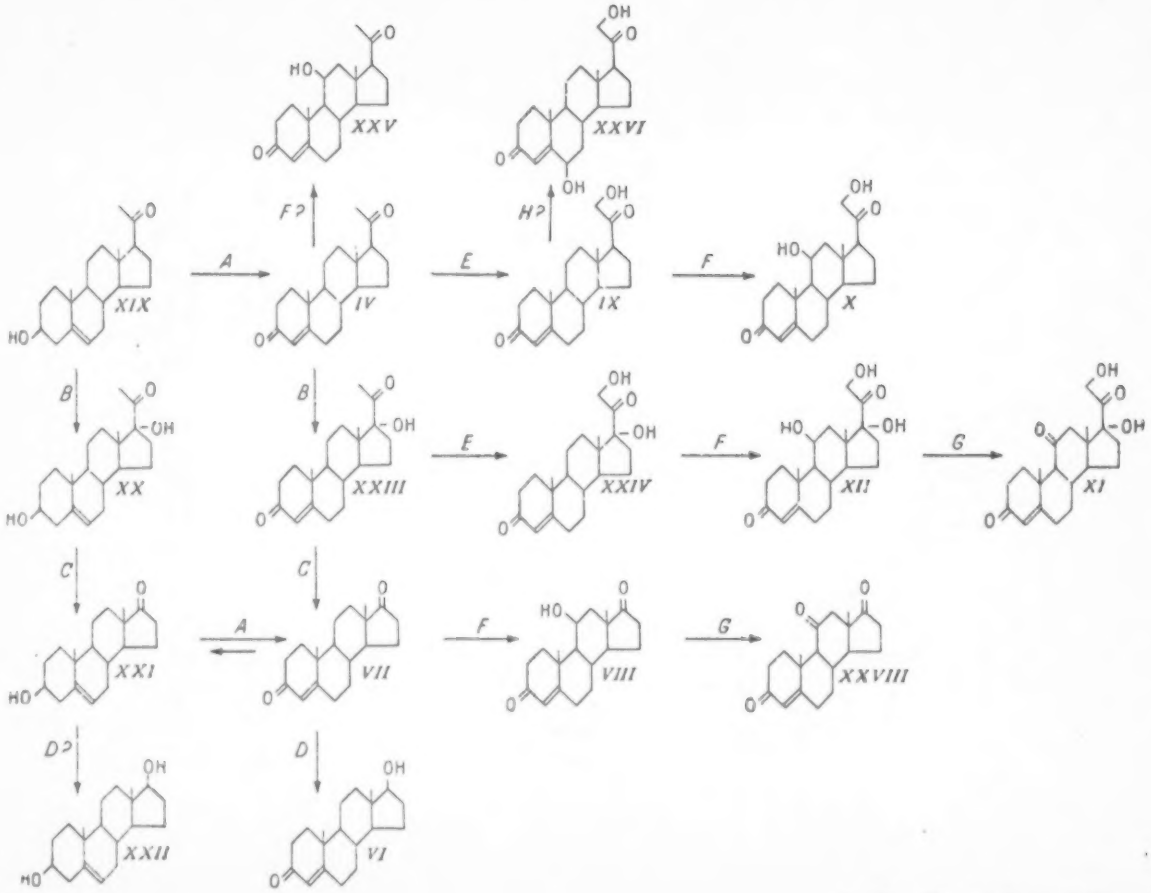
Isotope experiments prove that cholesterol can be a precursor of the non-benzenoid steroid hormones. BLOCH in 1945 administered deuterium-labelled cholesterol to a pregnant woman and recovered significant quantities of the isotope in the sodium pregnanediol glucuronidate isolated from the urine. He calculated that approximately 35 per cent of the material excreted during the 24-hr. period had originated from the cholesterol given. This was considered to be evidence of the conversion of the exogenous sterol to progesterone since pregnanediol excretion had been shown to increase after injection of this hormone (VENNING and BROWNE, 1937). Recently ZAFFARONI and co-workers (1951) perfused isolated bovine adrenals with C^{14}-cholesterol and found radio-activity in the 17-hydroxycorticosterone and cortisone recovered from the perfusate. Cholesterol, therefore, can be a precursor of at least some steroid hormones.

CLAESSON and HILLARP (1947) and FALCK (1953) noted a connection between the deposition of cholesterol in the interstitial cells of the ovary and evidence of estrogenic activity in the rat. CLAESON *et al.* (1948) thought that this relation indicated that cholesterol is a precursor of estrogens in the ovary. The evidence is at most only suggestive and is not supported by isotopic studies.

HEARD *et al.* (1954) injected a mare with a large dose of 4-C^{14}-cholesterol and isolated both the neutral and phenolic steroids. While the neutral steroids gave a count of 67,875 cts. per min. and the ketonic steroids had 30,000 cts. per min., estrone had no activity. Since, as previously mentioned, activity was found in estrone after the injection of radioacetate it seems doubtful that cholesterol is a significant intermediate in estrogen synthesis.

Even though cholesterol can be a precursor of nonbenzenoid steroid hormones, this does not prove that it is an obligatory intermediate. ZAFFARONI *et al.* (1951) and HECHTER *et al.* (1953b) compared the activities of the different cholesterol fractions and of 17-hydroxycorticosterone after the perfusion of isotopic acetate and isotopic cholesterol through bovine adrenal glands. The activity of the hormone was only about 0·4 that of the adrenal free cholesterol after the cholesterol perfusion, but was 6·4 times that of the free cholesterol after acetate perfusion. They postulate that there may be an intermediate between acetate and the hormone which is also involved in cholesterol synthesis and degradation.

Several factors make the experiment inconclusive on this point, however.



Caption Fig. 4. (*See opposite.*)

First, the steroid was isolated from the perfusate while the cholesterol fraction which would be the precursor was isolated from the gland. There is no way, therefore, to know the activity of the 17-hydroxycorticosterone being released from the gland at the moment the activity of the cholesterol was measured. Since no time study was run, it is impossible to know whether activity in the fractions was rising or had reached equilibrium. The postulate of a common intermediate on the basis of the greater activity of the hormone also includes a rather improbable assumption: that all of the free cholesterol is in the same metabolic pool as the hormone. Considering the distribution of cholesterol and its solubility characteristics, this seems unlikely. If a minor proportion of the free cholesterol (or of cholesterol ester) were an obligatory intermediate in the synthesis of the hormones and the balance were relatively inert, the results would be similar to those obtained in this experiment.

On the basis of his experiments BRADY (1951) also questioned the necessity of cholesterol as an intermediate in testosterone synthesis. While acetate was incorporated into cholesterol during incubation with testis slices, the radioactivity of the sterol was not so great as the probable activity of the testosterone and did not increase when chorionic gonadotropin was added to the incubation mixture while the amount of activity in the testosterone spots increased about tenfold. Since the slices were from normal testes, however, a large proportion of the cholesterol probably was in the germinal epithelium rather than in the interstitial cells (PERLMAN, 1951), and therefore definitely out of the metabolic pool in equilibrium with testosterone. Also the argument based on the differential effect of gonadotropin is open to question since relative specific activities were not determined, and the greater amount of activity may simply have represented increased amounts of testosterone of the same specific activity. Thus, the work which raises questions regarding the obligatory character of cholesterol as an intermediate in the synthesis of the steroid hormones is inconclusive.

Fig. 4. Enzymic reactions involved in the biosynthesis of the nonbenzenoid steroid hormones from pregnenolone.

IV: progesterone; VI: testosterone; VII: 4-androstene-3,17-dione; VIII: 11β-hydroxyandrostenedione (4-androsten-11β-ol-3,17-dione); IX: 11-desoxycorticosterone; X: corticosterone; XI: cortisone; XII: 17-hydroxycorticosterone; XIX: pregnenolone (5-pregnen-3β-ol-20-one); XX: 17-hydroxypregnenolone (5-preguen-3β,17α-diol-20-one); XXI: dehydroepiandrosterone (5-androsten-3β-ol-17-one); XXII: 5-androstene-3β,17β-diol; XXIII: 17-hydroxyprogesterone (4-pregnen-17α-ol-3,20-dione); XXIV: 17-hydroxy-11-desoxycorticosterone (4-pregnene-17α,21-diol-3,20-dione); XXV: 11-hydroxyprogesterone (4-pregnen-11β-ol-3,20-dione); XXVI: 6-hydroxy-11-desoxycorticosterone (4-pregnene-6β,21-diol-3,20-dione); XXVIII: adrenosterone (4-androstene-3,11,17-trione).

Probable distribution of enzymes involved in biosynthesis of hormones.

	Enzymes	
	High	Low*
Adrenal Cortex	A, B, E, F.	C. F†, G, H†
Testis	A, B, C, D.	E. F, G.
Corpus Luteum and Placenta	A.	B, E, F.

* No direct evidence. Based on small amounts of products seemingly requiring the specific action.

The steps by which cholesterol might be converted into steroid hormones are unknown at this time. All the tissues which form these hormones contain both C_{27} derivatives of cholesterol and C_{21} steroids, but no compounds with intermediate numbers of carbon atoms. This may signify that the six carbon atoms are split off as a unit.

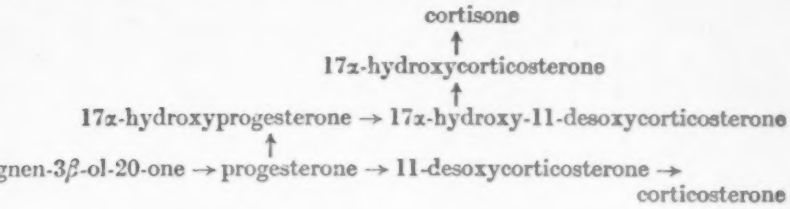
SAMUELS *et al.* (1951) reported that all tissues which secreted non-benzenoid steroid hormones contained enzymes which would oxidize a 3β-hydroxy compound to a 3-ketone (Enzyme A, Fig. 4) in the presence of diphosphopyridine nucleotide (DPN). Thus, 5-pregnen-3β-ol-20-one (XIX) would be converted to progesterone, or dehydroepiandrosterone (XXI) would be converted to 4-androstene-3,17-dione (VII). The enzymic activity seemed qualitatively the same in all the tissues: testis tissue would form progesterone from pregnenolone and adrenal tissue would form 4-androstene-3,17-dione from dehydroepiandrosterone. A wide range of other tissues was tested for this enzymic activity with negative results. It appeared that this was a general step in the synthesis of the steroid hormones. HELMREICH *et al.* (1953) studied the substrate specificity of the system. Only a 3β-hydroxy group will be oxidized. The ring structure must be flat (androstane or allopregnane nucleus, or Δ^5-unsaturated structure) and a ketone or hydroxyl group must be present at either C-17 or C-20. BEYER and SAMUELS (1954) have investigated the distribution of the enzyme within the cells of the adrenal cortex. It was found predominantly in the microsomes.

C. Late steps in biosynthesis

Though the early steps in steroid hormone biosynthesis are only dimly understood, the further conversions of the 21 carbon skeleton are fairly well worked out.

1. *Biosynthesis of adrenal hormones:* HECHTER and co-workers (1949) demonstrated that when bovine adrenal glands were perfused with blood containing ACTH, the perfusate contained increased amounts of several steroids known to occur in the adrenal glands. The greatest changes were in the amounts of 17α-hydroxycorticosterone (XII) and corticosterone (X). They then perfused the glands with various steroids which might conceivably be intermediates (HECHTER *et al.* 1951). When 5-pregnenolone (XIX) was added to the perfusate increased amounts of progesterone (IV), corticosterone (X), and 17α-hydroxycorticosterone (XII) were found. Progesterone yielded 17α-hydroxyprogesterone (XXIII), corticosterone, 17α-hydroxycorticosterone, and small amounts of 11β-hydroxyprogesterone (XXV). 17α-Hydroxyprogesterone yielded only 17α-hydroxycorticosterone, and 11-desoxycorticosterone (IX) yielded only corticosterone. 17α-Hydroxy-11-desoxycorticosterone (XXIV) gave only 17α-hydroxycorticosterone. A series of reports from this group on the products obtained after perfusion of beef adrenals with various steroids has appeared recently (JEANLOZ *et al.*, 1953; LEVY *et al.*, 1953; MEYER, 1953a, 1953b; MEYER *et al.*,

1953a, 1953b). On the basis of these results the investigators postulated the following sequence of reactions in the adrenal glands:



These workers concluded that hydroxylation of carbon-21 prevented the introduction of a hydroxyl group at position 17; thus two major products resulted, corticosterone and 17-hydroxycorticosterone, the proportion depending on the relative activity of the 17-hydroxylating system.

Soon thereafter the oxidation of carbon-11 by adrenal slices and homogenates was demonstrated by several groups of workers (Enzyme F). Hayano *et al.* (1949), Savard *et al.* (1950), McGinty *et al.* (1950), and Kahnt and Wettstein (1951) all were able to convert 11-desoxycorticosterone (IX) to corticosterone (X) and 17-hydroxy-11-desoxycorticosterone (XXIV) to 17-hydroxycorticosterone (XII) in such preparations. Haines (1952) also obtained 6β-hydroxy-11-desoxycorticosterone (XXVI) from 11-desoxycorticosterone. The 11-keto derivatives were isolated in small amounts by several of the groups.

Not only could added substrates be oxidized at carbon-11 by tissue minces and homogenates, but the formation of both corticosterone and 17-hydroxycorticosterone from endogenous substrates was also demonstrated in such preparations. Saffran *et al.* (1952) did not find much formation by adrenal slices alone, but when ACTH was added the production of glycogenic corticoids during incubation of adrenal slices was markedly increased. Since the rate of formation was related to the concentration of ACTH over a considerable range, they recommended this incubation as an assay for the pituitary hormone. Hoffman *et al.* (1952) incubated fresh adrenal homogenates in a medium containing 1 per cent glucose and found measurable formation of both corticosterone and 17-hydroxycorticosterone. The addition of either fumaric acid or ascorbic acid approximately doubled the yield. The action of the two was different: fumaric acid increased the oxygen consumption of the tissue and primarily enhanced the 11-oxidation; ascorbic acid was effective in the absence of oxygen and did not specifically increase oxidation at carbon-11. When both were included, the action appeared additive.

Sweat (1951) fractionally centrifuged homogenates of beef adrenals in 0·88 M sucrose and found that all of the 11-hydroxylating activity was present in the mitochondria. Using 17-hydroxy-11-desoxycorticosterone as substrate, he found that fumarate was necessary and that the optimum *p*H was near 7. He did not find that adenosinetriphosphate (ATP) enhanced the activity. Hayano *et al.* (1951) independently confirmed the presence of the 11-hydroxylating enzyme in the mitochondria.

A more extensive study by HAYANO and DORFMAN (1953) has more completely characterized the 11-hydroxylating enzyme system. When washed fresh adrenal mitochondria were used as the source of the enzyme only Mg and fumarate were essential for the reaction, as originally stated by SWEAT. They also confirmed SWEAT regarding optimum *p*H, finding it to be 7·4 when free desoxycorticosterone was used as substrate. When they used mitochondria prepared from glands stored for several weeks in the freezer, however, they found that DPN and ATP were also necessary. Apparently these were sufficiently firmly attached to the mitochondria to survive the original washing but were destroyed in the stored glands.

Substrate specificity was also studied. To react, a C_{21} steroid must have an alcohol group at C-21 and a ketone group at C-20. Reduction of the Δ^4-double bond decreases the rate of reaction somewhat. Conversion of the ketone group on carbon 3 to an alcohol markedly decreases the rate and the additional reduction of ring A to the saturated alcohol practically stops the reaction. If the α,β unsaturated system is present in C_{19} compounds, together with a ketone group at carbon-17, the reaction will go rather rapidly. Reduction of the 17-ketone to a 17-hydroxy group decreases the rate, and reduction of ring A as in androsterone prevents the reaction.

In 1952 PLAGER and SAMUELS reported the hydroxylation of C-21 by adrenal homogenates (Enzyme E). Both the enzyme system involved in this reaction and that hydroxylating C-17 (Enzyme B) were described by these workers in 1953 (PLAGER and SAMUELS, 1953a). These enzymes were in the supernatant after the mitochondria were centrifuged down, thus preventing hydroxylation at C-11. When progesterone was incubated with such preparations the products were 11-desoxycorticosterone and Compound S of Reichstein (17-hydroxy-11-desoxycorticosterone, XXIV). When desoxycorticosterone was incubated with the supernatant, however, no Compound S was formed. Either compound when incubated with adrenal mitochondria yielded its respective C-11 hydroxylated derivative but not that of the other. Thus the sequential relations indicated by HECHTER seem to be confirmed by the studies of individual enzyme systems. Sequential incubation with supernatant and mitochondrial preparations was used to convert 4-C^{14}-progesterone to 4-C^{14}-17-hydroxycorticosterone (PLAGER and SAMUELS, 1953b).

The series of oxidations just described does not account, however, for all of the products of the adrenal glands. HECHTER *et al.* (1951) found 5 spots on their chromatograms of adrenal perfusates which were more polar than 17-hydroxycorticosterone. These were present in the blood before perfusion but were increased threefold when the blood containing ACTH was passed through the isolated bovine glands. BUSH (1951) reported that such unknown compounds often made up the greater portion of the steroids in dog adrenal vein blood. He also found three such spots in chromatograms of the "amorphous fraction" from adrenal extracts (BUSH, 1952). These compounds absorbed ultraviolet light and gave yellow fluorescence with sodium hydroxide, indicating a Δ^4-3-ketone structure. These same spots were observed in chromatograms of

human peripheral blood by BUSH and SANDBERG (1953). HAYNES *et al.* (1953), found that incubation of 17-hydroxycorticosterone with adrenal slices led to the formation of similar compounds. When cortisone was incubated with the slices, only the least polar of the three spots was formed. These compounds have not yet been identified. Some of these may be 6β-hydroxy compounds since hydroxylation at this position has been shown to be catalyzed by adrenal tissue (HAINES, 1952).

No one thus far has demonstrated the formation of 17-ketosteroids from C_{21} steroids in the adrenal glands. As will be discussed later, at least one other gland contains enzymes which will catalyze this reaction, however, and it may be that the system has not been looked for in the proper way.

2. *Biosynthesis of testicular hormones:* When RUZICKA and PRELOG (1943) isolated 5-pregnen-3β-ol-20-one (XIX) from testes its relation to the known androgens was obscure. Recent work has demonstrated that, as in the case of the adrenal cortex, the reactions in the testis can be followed from this compound to the known secretory products.

As already mentioned, SAMUELS *et al.* (1951) had been able to convert pregnenolone into progesterone with testis homogenates; dehydroepiandrosterone was also converted to androstenedione. They were not able, however, to identify any conversion of C_{21} steroids to C_{19} compounds. This apparently was because the conditions used were not optimal for the subsequent steps which we now know to take place.

SLAUNWHITE and SAMUELS (1954) incubated 4-C^{14}-progesterone with slices of testis from hypophysectomized rats which had been injected with chorionic gonadotropin. Thus the testicular tissue consisted largely of interstitial cells and connective tissue. When the lipid extract of the incubated material was chromatographed on paper by the BUSH technique (BUSH, 1953b), a number of radioactive spots was identified in addition to that of progesterone. One moved in two different systems with the same R_f value as 17α-hydroxyprogesterone (XXIII). Another showed the same chromatographic characteristics as 4-androstene-3,17-dione (VII) while a third moved like testosterone (VI). Another radioactive spot moved just ahead of corticosterone but has not been identified further. There was also radioactive material which was more polar than 17-hydroxycorticosterone. When 21-C^{14}-progesterone was used as substrate a small amount of the isotope was found as carbon dioxide and much larger amounts were in the steam distillate of the acidified aqueous solution. It would appear, therefore, that the testis has enzymes similar to those in the adrenal cortex, except that those which hydroxylate positions 11 and 21 are relatively inactive while that hydroxylating carbon-17 is quite active. It also has relatively high amounts of an enzyme (Enzyme C) which will split off the side chain of the 17-hydroxylated compounds to yield C_{19} compounds and short chain acids (see Fig. 4).

If the sequence is as indicated, why have not progesterone and 17α-hydroxyprogesterone been isolated from testis extracts? Probably because steps A and

B in Fig. 4 are the slow steps in the synthesis and the products are further converted before significant amounts accumulate.

3. *Biosynthesis of hormones of corpus luteum and placenta:* Apparently the corpus luteum has relatively high concentrations of 3β-ol dehydrogenase (SAMUELS *et al.*, 1951). Thus its product is primarily progesterone. That other systems similar to those in the testis are present in low activity, however, is indicated by the work of KATSH (1950). He transplanted dissected corpora lutea and ovaries into the seminal vesicles of castrated male rats and found androgenic activity associated with luteinization. Also, when bovine corpus luteum tissue was incubated with 11-desoxycorticosterone, 6β-hydroxy-11-desoxycorticosterone (XXVI) was isolated (HAYANO *et al.*, 1953). This is the same compound found by HAINES (1952) as one product of the incubation of desoxycorticosterone with adrenal slices. Probably low activities of other enzymes found in the adrenals will be discovered.

The reactions by which the placenta synthesizes its steroid hormones are poorly worked out. SAMUELS *et al.* (1951) demonstrated the presence of the 3β-ol dehydrogenase in this tissue. The isolation of 3β-hydroxy derivatives of allopregnane and pregnene by PEARLMAN and CERCEO (1952, 1953) indicates that precursors similar to those in the other glands are available. The recent report of JOHNSON and HAINES (1952) that a small amount of adrenal-like metabolic activity is present in lipid extracts of the placenta, together with the evidence that the levels of plasma 17,21-dihydroxy-20-ketosteroids rise steadily during pregnancy to about four times the normal value (GEMZELL, 1953), raises the possibility of the presence of low activities of certain of the hydroxylating systems. This is supported by the work of HAYANO *et al.* (1953) who found evidence of a more polar reducing substance after incubation of 11-desoxycorticosterone with beef placenta. Much needs yet to be done on this tissue which can produce such large quantities of both C_{21} and C_{18} compounds.

4. *Biosynthesis of estrogens:* No work on the synthesis of estrogens from other steroids *in vitro* has been reported. The chemical work with radioacetate has already been mentioned. After injection of 1-C^{14}-acetate, HEARD and coworkers (1954) found that the specific activity of estrone was about twice those of the two more unsaturated compounds, equilin and equilenin. Equilenin was somewhat less active than equilin. When C^{14}-estrone was injected no activity was found in the more unsaturated estrogens. It seems probable, therefore, that estrone is not a precursor of equilin and equilenin.

5. *Summary:* As the results stand at present, it seems that the nonbenzenoid steroid hormones are formed by a similar series of reactions, the differences being in relative activities of certain enzymes involved in the later stages of synthesis. Thus, they appear to be formed from 2-carbon units, perhaps *via* cholesterol. The α,β unsaturated ketonic system in ring A is common to all these hormones. Its formation is due to an enzyme requiring DPN as hydrogen acceptor which seems to be the same, or very similar, in all the sites of synthesis.

In the adrenal cortex there are enzymes which carry out hydroxylations at positions 17, 21 and 11, and perhaps others. In the testis the 17-hydroxy

enzyme and a side chain-splitting enzyme predominate, while in the corpus luteum and placenta, catalysis of oxidations other than that in ring A is relatively small. When proper conditions are achieved, however, small activities of nearly all the hydroxylases can be demonstrated in all these tissues.

The synthesis of the estrogens, while perhaps resembling that of the non-benzenoid steroids in the early stages, apparently is not simply a further unsaturation of the non-benzenoid steroids. In the mare, at least, the aromatic rings seem to be derived from sources other than acetate, and cholesterol does not seem to be involved. Further, the more unsaturated estrogens, equilin and equilenin, do not seem to be formed from estrone. Much needs to be done with the *in vitro* systems to unravel this problem.

III. Transport of Steroid Hormones

As pointed out in the introduction, one criterion of a hormone is that it is transported from the gland of origin to the target cells by the blood. We have already cited the evidence for the presence of increased concentrations of certain compounds in the blood flowing from the adrenal glands, testes and placenta. In the case of the adrenal hormones these compounds have been measured in the peripheral circulation (Nelson and Samuels, 1952; Sweat and Farrell, 1952; Morris and Williams, 1953; Bush and Sandberg, 1953), and the androgenic, estrogenic and progestational activities of the peripheral blood have been determined biologically (Womack and Koch, 1932; Fluhman, 1934; Szego and Roberts, 1947; Hooker and Forbes, 1947).

These compounds seem easily extractable, yet the free steroids (not esterified) of the urine are practically inactive when tested biologically; only after acid or enzymic hydrolysis are significant amounts of active compounds obtained. Even then, in the case of the non-benzenoid hormones, only traces of the original steroids are found. Most of the urinary steroids are not found in the endocrine glands. This conservation of the steroid hormones is due in large part to their ability to form complexes with the plasma proteins. Here again, as in synthesis, the non-benzenoid group appears to differ from the estrogens.

The steroids of the adrenal cortex, testis and corpus luteum appear to be only loosely bound in the blood since they can be readily extracted therefrom with lipid solvents (Vogt, 1943; Reich *et al.*, 1950; Bush, 1951; Bush and Sandberg, 1953; West *et al.*, 1952; Hooker and Forbes, 1949). They appear to be almost entirely in the plasma (Hooker and Forbes, 1949; Bush, 1951; Nelson and Samuels, 1952, Zander, 1954), but may penetrate the cells when drawn blood is allowed to stand (Eik-Nes, 1953).

Bischoff and Pilhorn (1948) were the first workers to demonstrate clearly that these compounds are not wholly free, but combine loosely with serum proteins. They showed that testosterone, progesterone and estradiol are more soluble in blood serum than in physiological salt solutions. The increase in solubility was almost as great if serum albumin solutions were used which were equivalent in concentration to the albumin in the serum. The globulin fractions of bovine serum had little influence on steroid solubility.

Eik-Nes and co-workers (1954) have studied this combination of steroids with serum albumin. Both dialysis and solvent partition experiments show that the bound steroid is in equilibrium with the free steroid dissolved in the aqueous solvent. The binding is in part electrostatic, increasing with the number of polar groups. Position seems important since binding is particularly increased by the presence of polar groups at each end of the planar steroid molecule, approximately 20 Å apart. Ionic strength has little effect, the binding of the steroid apparently being much stronger than that between serum albumin and such ions as chloride. The binding force, but not the number of binding groups, increases markedly between *p*H 6 and 9, probably indicating the involvement of imidazol groups on the protein (Schellman *et al.*, 1954). On the other hand, a large portion of the binding energy appears to be due to van der Waals forces. Schellman *et al.* interpret these facts as indicating that the binding involves the breaking of specific tertiary bonds in the folded albumin molecule with the steroid acting as a bridge between the separated groups.

While the binding of steroids is increased with increasing polarity, the effect is not so great as on the binding of water. Consequently, when an aqueous serum albumin solution is saturated with a more polar steroid, the total amount dissolved is greater but the ratio of bound to free steroid is lower. Thus more free 17-hydroxycorticosterone is found in the urine compared with its concentration in plasma than are any of the other non-benzenoid steroid hormones.

The free steroids of adrenocortical origin in peripheral blood are similar to those reported in the adrenal vein blood except for proportionately higher amounts of the 11-keto derivatives. Bush and Sandberg (1953), using paper chromatography, identified 17-hydroxycorticosterone as the major component, with cortisone next (about 1/5 the concentration), and corticosterone only appearing irregularly. In addition there were 3 spots absorbing ultraviolet light which moved more slowly than 17-hydroxycorticosterone and one which moved near the R_f of corticosterone but which was not this steroid. Sweat *et al.* (1953) using partition chromatography on silica gel and measurement by sulphuric acid fluorescence, found 4·2 to 5·8 μgm per 100 ml plasma of a compound behaving like corticosterone and 7·8 to 12·8 μgm per 100 ml plasma of 17-hydroxycorticosterone. Morris and Williams (1953), using column chromatography and polarographic estimation, reported more than half as much corticosterone as 17-hydroxycorticosterone. In addition, they found about the same proportion of cortisone as Bush and Sandberg, and an equal amount of 11-dehydrocorticosterone. This seems to indicate that oxidation of the 11β-hydroxy group on the hormones originally secreted takes place somewhere outside the gland of origin.

The discrepancy between the findings of Bush and Sandberg and the other two groups regarding the regularity of occurrence and the amount of corticosterone is at present unresolved. The former workers point out, however, that on their paper chromatograms there is an unknown substance moving

very near corticosterone which might not be separated by the two types of column chromatography used by the other groups, and which might give fluorescence and a polarographic wave similar to this steroid. Final decision regarding the quantitative relation of corticosterone in human peripheral blood must await better identification of the material being measured.

Thus far no one has succeeded in measuring the levels of free testicular hormones in the peripheral blood. Conjugated 17-ketosteroids have been determined but these will be discussed under catabolism of the steroids.

The form of the circulating progestins is still uncertain. HOOKER and FORBES (1949) and FORBES (1950, 1951), using their rather specific bioassay, have reported relatively high concentrations, equivalent to 1 to 8 μgm progesterone per ml plasma, in human, monkey, and rabbit blood during the luteal phase of the cycle and in pregnancy. As in the case of the cortical steroids, the material appears to be almost entirely in the plasma and in the free form. It has already been noted that EDGAR (1952), using paper chromatography, has identified progesterone in the ovarian vein blood of the ewe and BUTT *et al.* (1951b) have obtained polarographic evidence of it in placental blood. Recently ZANDER (1954), using an elegant micromethod (ZANDER and SIMMER, 1954), not only succeeded in measuring progesterone in the peripheral blood during the last half of pregnancy, but actually isolated a sufficient amount of the pure compound to establish its identity by infrared spectra. The levels found, 4–29 μgm per 100 ml plasma, were much below those reported using the bioassay of Hooker and Forbes; it would seem, therefore, that either the biological action of progesterone is potentiated by some substance in the blood extracts or there is also some other active compound present. In any case, both the biological and chemical data fit with the concept of a steroid hormone bound to protein but in equilibrium with the free steroid in solution.

As in the case of synthesis, the estrogens appear to be in a class apart, the benzene ring conferring new properties on the steroid molecule. SZEGO and ROBERTS have studied the characteristics of the circulating estrogens, using the uterine weight assay of ASTWOOD (1938). Approximately two-thirds of the estrogenic activity is not extractable with organic solvents but can be separated completely by dialysis; the dialysate, however, must be subjected to acid hydrolysis for recovery (SZEGO and ROBERTS, 1946; ROBERTS AND SZEGO, 1946). The bound material was found entirely in the β-globulin fraction III-0 of COHN *et al.* (1946). The estrogen obtained after acid hydrolysis had the solubility characteristics of estriol. They therefore concluded that the circulating estrogen was estriol conjugated with some hydrophilic group which formed an equilibrium complex with a specific β-lipoglobulin.

ROBERTS and SZEGO (1947) observed that the response of the immature rat uterus to an intravenous dose of estrogen was markedly reduced by evisceration or partial hepatectomy. On the other hand, the response was significantly greater than normal during rapid liver regeneration. On the theory that the estroprotein complex might be formed in the liver, and that this was the active form of the hormone, they carried out incubations of 16-C^{14}-estradiol with liver

tissue *in vitro* in the presence of blood serum. The serum-bound estrogen increased. When other tissues were substituted for liver no such increase in bound estrogen was observed. Regenerating liver was more effective than normal liver. The isotopic steroid could be dialyzed from the protein but could not be extracted with ether until the dialysate had been heated with strong acid (SZEGO, 1953). As a result of these experiments, SZEGO and ROBERTS (1953) postulate that the estrogens are secreted from the ovaries as the free steroids but are conjugated with a hydrophilic group in the liver which is bound to the III-0 fraction of the β-globulins, and that this estroprotein is the form in which the steroid is transported to the target cells. The conclusive experiment, injection of the biosynthesized estroprotein into an eviscerated ovariectomized female rat, has not been reported.

The blood is not an entirely inert medium as far as the steroids are concerned. BISCHOFF and co-workers (1951) have shown that red cells contain systems which will hydrolyze conjugated estrogens. Free estrone was converted into biologically more active material, probably estradiol (BISCHOFF *et al.*, 1952). They also report that rabbit red cells can oxidize the 17-ol group of testosterone or reduce the 17-ketone of 4-androstene-3,17-dione (BISCHOFF *et al.*, 1953b). HOOKER and FORBES (1949) reported that the progesterone-like activity determined by their bioassay disappeared rapidly in plasma stored at 37°, but EDGAR (1953), using a chemical assay, could not confirm this. This is another observation which suggests that the bioassay is not dependent on progesterone alone.

MEYER (1953a) was able to recover an average of only 78 per cent of added cortisone when bovine blood was aerobically incubated for 3 hours. After incubation the extracts showed spots corresponding to the tetrahydro compounds formed by reduction of ring A to a saturated alcohol. EIK-NES (1953) had also observed disappearance of 17-hydroxycorticosterone on incubation of plasma at body temperature. The reaction has a high temperature coefficient, being very slow at 22° and insignificant at 2°. It may well be that similar reactions occur with the other steroid hormones; they have not yet been studied. While such reactions probably occur slowly in the blood under physiological conditions they do not play a major role in the metabolism of the hormones. Other tissues are much more active in the inactivation and removal of hormones.

IV. Catabolism of the Steroid Hormones

A. Introduction

The inactivating metabolism (or catabolism) of the non-benzenoid steroid hormones was reviewed recently by SAMUELS and WEST (1952) and the material covered therein will be only briefly dealt with as a framework for understanding the more recent literature.

The large number of steroids isolated from human urine (ROMANOFF *et al.*, 1953b), most of which are not known to be secreted by an endocrine gland, bears

eloquent testimony to the many reactions which these compounds undergo in the animal body. No attempt will be made to list all of the steroids isolated from urine which *might* be connected with specific hormones; only those will be mentioned which have been definitely shown to increase after injection of a specific hormone. Neither will those steroids be considered which have been found in increased quantity in the urine of patients with tumors of the endocrine glands since in many cases the symptoms produced indicate abnormal function, and this may be true in greater or lesser degree for all. Often the products in the urine appear to be metabolic products of substances which are found in extracts of the gland but which have not been demonstrated to be secreted. These precursors may escape into the circulation normally in small amounts but, in many cases, their major function seems to be as intermediates in the synthesis of the known hormones. Many have no known physiological action and therefore cannot be considered hormones; their excretion products, consequently, do not fall within the scope of this review. A masterly consideration of these substances and their probable relationships is given by LIEBERMAN and TEICH (1953).

The most widely used method of studying the catabolism of the steroid hormones has been the isolation and measurement of urinary steroids after the administration of large amounts of hormone. This has many drawbacks. The increased output of a given steroid may be due to the effect of the injected compound on the metabolism of steroids from some other source. Even though the compounds measured in the urine do come from the metabolism of the administered substance, the ratios of one product to the other cannot be interpreted as normal since they are produced in the presence of excess substrate, and different enzyme systems may become limiting. Then too, only one route of excretion is examined, and it is rare to recover as much as 50 per cent of the administered dose. In spite of this, important information has been obtained. The list of steroids found in increased amount in the urine after the administration of the hormones indicates that the major reactions are reductive. That oxidative reactions also occur is plain from the fact that androsterone and etiocholan-3α-ol-17-one, which have a ketone group on carbon-17, are the compounds which are increased most after the administration of testosterone. It is also obvious that conjugation plays an important role since almost all of the steroids in the urine are combined rather firmly with groups which render them water-soluble.

Recently the preparation of steroid hormones labelled with isotopes has permitted a great improvement in this technique. Doses can be used which are within the estimated normal level, and more than one route of excretion can be examined. When carbon is used as the isotope, any labelled compound can be considered a product of the metabolism of the hormone, since when we refer to metabolic products it is ordinarily understood that we mean the carbon skeleton. The information obtained by overall experiments, even with isotopes, still leaves much to be desired, however, since nothing is known of the routes by which the changes were brought about, or where they occurred.

We have already seen that some changes can occur in the blood itself, but

these are relatively slow and could not account for the rapid conversions after administration of certain steroids. We must, therefore, look elsewhere for active enzyme systems. To know where steroids undergo changes in the body three techniques have been used. In one the steroid is implanted in an area where the blood flows to one major organ and the biological effect is compared with that of a similar implant in an area where the blood enters the general circulation. A second is the perfusion technique, used so effectively in exploring the process of biosynthesis. The third is the use of isolated tissue slices, homogenates, or protein fractions. This method permits a more detailed study of an individual reaction but tells only what can happen. Its significance in the metabolism of the steroids in the intact animal can only be judged by comparison with the overall excretion studies and the results of implantation. The results of tissue studies indicate that the liver appears to be the major site of these reactions, with the kidney playing the next most prominent part, and other tissues also showing activity.

B. Metabolism of estrogens

Until recently, most of the work on the metabolism of the estrogens depended on biological assay for measurement of change. This was because of the very low concentrations in biological material under normal conditions. The phenol-sulphonic acid reaction of KOBER (1931) and its various modifications made possible the chemical measurement of estrogens. It was applied to the estimation of estrogens in the urine of pregnancy by VENNING *et al.* (1937), but could not be used for the estimation of the small quantities excreted during the normal menstrual cycle. SZEGO and SAMUELS (1943a) applied a modification of the original method to the study of the interconversions of the estrogens by placental and uterine tissue. LIEBERMAN *et al.* (1952) developed a method involving the coupling of a chromophore group with the phenolic hydroxyl of the estrogens for studies with liver tissue.

It was not until BATES and COHEN (1950) called attention to the intense fluorescence of estrogens in the presence of concentrated sulphuric acid alone, that it became feasible to measure amounts similar to those normally excreted by the mature woman. Unfortunately fluorescence in strong sulphuric acid is not a reaction limited to the estrogens; it is quite non-specific. Highly specific methods of isolation were necessary before the technique could be applied to biological material. While considerable progress has been made, there is no system of purification yet where the bioassay of the product is not lower than the fluorescence value. The best methods are the countercurrent procedure of ENGEL *et al.* (1950), and the paper chromatography techniques (BUSH, 1953b). These, together with the use of isotopic steroids, should help in the elucidation of estrogen metabolism. Much of the work, however, depends on bioassay, and can only be interpreted chemically from solubility data and knowledge of alterations in structure which destroy estrogenic activity.

1. *Overall metabolism:* From human urine the compounds estrone (I), 17β-estradiol (III) and estriol (1,3,5-estratriene-3,16β,17β-triol) (II) have been

isolated. In addition, 17α-estradiol (XXIX), equilin (XXX) and equilenin (XXXI) have been found in pregnant mares' urine (Fig. 5).

Only traces occur as free compounds, most being present as conjugates. Estrone sulphate was isolated from mares' urine by SCHACHTER and MARRIAN (1938), and estriol glucuronidate was isolated from human pregnancy urine by COHEN and MARRIAN (1936). Estrone glucuronidate was found by ONESON and COHEN (1951) to be the major conjugate of this steroid in the urine of

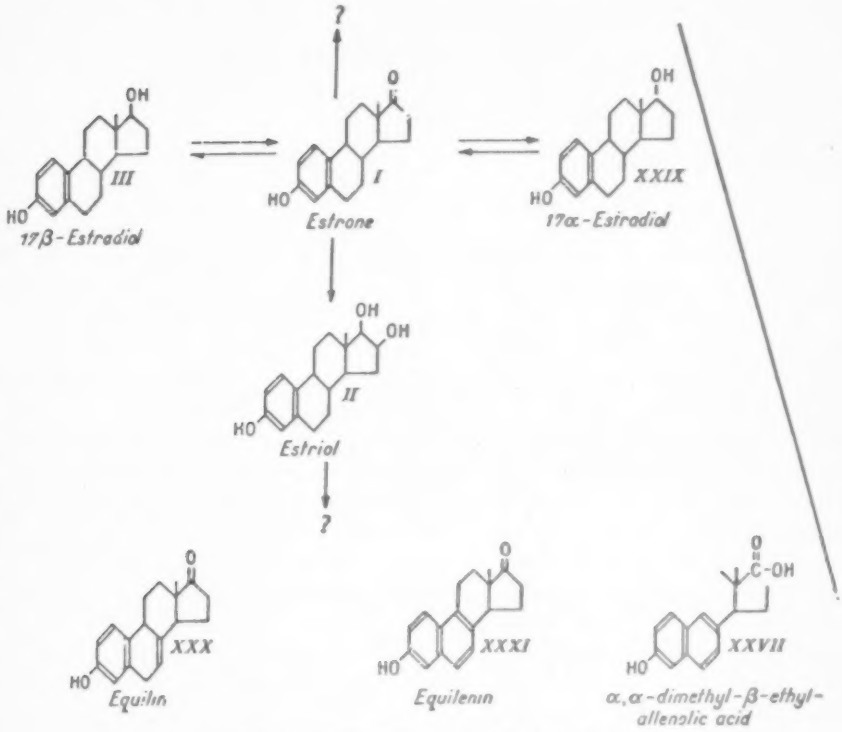


Fig. 5. Estrogens and proestrogens. Route of probable metabolism in the human and rabbit indicated.

pregnant women. BEER *et al.* (1953) administered 16-C^{14}-estrone and 16-C^{14}-17β-estradiol to women and found that most of the radioactivity in the urine was released by β-glucuronidase. A small amount was separated in the acidic fraction; the balance was phenolic. It seems, therefore, that glucuronic acid is the major hydrophilic group conjugated with estrogens in the human being.

When 17β-estradiol, the compound isolated from hogs' ovaries, is injected into humans of either sex, many workers have found that the amounts of estrone and estriol are increased. In rabbits increased amounts of 17α-estradiol have also been found. If estrone is injected, increased amounts of 17β-estradiol and estriol are found, and again in the rabbit, 17α-estradiol (STROUD, 1939).

When, however, estriol is injected, the only estrogen which is found in increased quantity is estriol itself (DOISY *et al.*, 1942; PINCUS and ZAHL, 1937; SCHILLER and PINCUS, 1943). None of the three other possible isomers of estriol (16α,17β; 16α,17α; or 16β,17α-) have been isolated from natural sources. On the basis of these overall studies the following general sequence has been postulated for mammals other than the equine:

$$17\beta\text{-estradiol} \rightleftarrows \text{estrone} \rightarrow \text{estriol-3,16}\beta\text{,17}\beta$$
$$\downarrow\uparrow$$
$$17\alpha\text{-estradiol}$$

The bile apparently plays an important role in the excretion of the estrogens. CANTAROW *et al.* (1943) found estrogenic activity in the bile of dogs and women. The concentration in the human bile was much greater than in samples of blood taken at the same time. Evidence for enterohepatic circulation was obtained when 17β-estradiol introduced into the intestine appeared in the bile. When BOCKLAGE *et al.* (1951) administered 17-C^{14}-methylestradiol to rats by stomach tube most of the material appeared in the bile during the subsequent two days; very little appeared in the urine. When the bile duct was ligated, however, most of the isotope was excreted as urinary steroids, but at a slower rate than in the intact animal.

DOISY *et al.* (1952) found that the radioactivity of 16-C^{14}-estrone likewise appeared primarily in the faeces and not in the urine. LEBLOND (1951) studied the distribution of isotope following the injection of this labelled steroid into mice. About 2 per cent appeared as CO_2 in the expired air, 20 per cent was in the urine; the balance recovered was in faeces and bile. After acid hydrolysis, 75 per cent of the activity in the urine was still water-soluble. The same was true of the faecal activity. Thus rupture of the cyclopentane ring and other extensive changes take place in the rodent, either in the tissues or by bacterial action in the gut.

To what extent the estrogens secreted in human bile are ultimately eliminated in the faeces remains to be determined. These observations indicate, however, that the rate of clearance of a steroid from the blood cannot be considered a measure of its conversion into other compounds unless biliary excretion of the original steroid has been ruled out.

2. *Metabolism by specific tissues:* The first evidence of the role of the liver in steroid metabolism was obtained by ZONDEK in 1934. He found that a short time after a given amount of estrogen was injected into a mouse only a small fraction of the biological activity could be recovered in extracts of the animal and its excreta. When the estrogen was incubated with mouse liver the activity was rapidly lost, but not when other tissues were employed. ZONDEK claimed to have prepared an acetone-dried powder which would cause this inactivation.

Following these initial observations, many workers demonstrated the major role of the liver in the inactivation of the estrogens. ISRAEL *et al.* (1937) introduced estrone into the blood of a heart-lung-liver preparation of a dog and found that the estrogenic activity disappeared rapidly from the circulation; when,

however, the liver was eliminated from the circuit there was no measurable loss. The activity which disappeared could not be recovered by extracting the liver. BISKIND and MARK (1939) implanted pellets of various estrogens in the spleens of immature female rats. As long as the spleen remained *in situ* no estrus resulted; when, however, the spleen was transplanted so that the venous blood drained into the general circulation instead of into the portal system, the animals immediately went into estrus.

HELLER (1940) reported that when he incubated estrone or estradiol with rat liver slices under aerobic conditions, biological activity was lost. If CN^- were introduced into the system, however, the biological activity of estrone was increased as much as twenty-fold while that of estradiol was unchanged. Since the maximum increase found was equivalent to the difference between the activities of estrone and of estradiol in the uterine weight assay, he interpreted this as indicating that estrone was converted to estradiol in the presence of cyanide but further conversion to inactive compounds was inhibited.

LIEBERMAN *et al.* (1952) used their colorimetric method for the estimation of estrogens to study metabolism of these steroids *in vitro*. The method gives almost equal colour intensity with estrone and 17β-estradiol but only about half the intensity with estriol. Incubation of rat liver slices with either estrone or estradiol led to marked loss of colour. Estriol was destroyed, but not at as high a rate as the other two. While some of the apparent destruction of estradiol and estrone may have been due to conversion to estriol, it would seem that some estrogen was converted to non-phenolic products. TAGNON *et al.* (1952) found that human liver also caused the same type of changes.

COPPEDGE *et al.* (1948) using bioassay have investigated the inactivation of estrogens by liver homogenates. Oxygen and DPN were essential. The latter appears to be the only effective hydrogen acceptor. In an attempt to determine the intracellular location of the enzyme system involved, RIEGEL and MEYER (1952) fractionated liver homogenates into nuclei, mitochondria, microsomes and supernatant fluid. They then studied the biological inactivation of 17β-estradiol and estrone by each fraction. All preparations were incubated aerobically in phosphate buffer containing nicotinamide with DPN as hydrogen acceptor. The whole homogenate was quite active, but none of the fractions alone was effective. When the heated supernatant was added to the microsomes, however, full activity was restored. The supernatant could be replaced by riboflavin monophosphate (RMP). Apparently the enzyme is in the microsomes, and requires both DPN and RMP for its action.

Not all of the reactions which the liver carries on are degradative, however; some are synthetic. The range of compounds which, when injected into a castrate female, will produce symptoms of estrus, is quite wide. Even a compound like triphenylethylene is active. The original assumption was that the locus of action in the target tissue was relatively non-specific. EMMENS (1941, 1942) compared the minimum effective dose of the various estrogenic compounds when applied directly to the uterus or vagina with that required subcutaneously. He found that the so-called estrogens could be divided into two groups: those

which were much more effective when directly applied, and those in which the direct dose was as large as the subcutaneous dose. He deduced that the latter must require metabolism in some other organ before they could act as estrogens —"proestrogens," while the former were the "true estrogens." Later, SEGALOFF (1948) confirmed this deduction in the case of triphenylethylene. Whereas much larger amounts of the true estrogens were required when introduced in the spleen than when given peripherally, the opposite was true with triphenylethylene. Thus it appeared that the liver was the tissue which converted the proestrogen into an active estrogen. Recently this has been demonstrated with α,α-dimethyl-β-ethylallenolic acid (XXVII) (COURRIER *et al.*, 1951). When this compound is fed to rats larger amounts of estrogenic activity are found in the excreta than would be attributable to the original compound. This is not due to bacterial action in the gut but depends on the presence of the liver. Apparently the original compound is absorbed, converted into estrogen as it passes through the liver, and considerable amounts of the estrogen formed are then excreted into the gut *via* the bile.

The liver is not the only tissue, however, which has been demonstrated to cause changes in estrogens *in vivo*. HELLER (1940), using an increase in biological activity as evidence, claimed that he obtained conversion of estrone to estradiol in the presence of uterine or lung tissue with or without cyanide, and concluded that these tissues contained a reductive system similar to that in the liver but lacked the oxidative enzymes inhibited by cyanide. BISCHOFF *et al.* (1953a), also using the immature rat uterus for assay, have confirmed HELLER's observations on the activity of uterus and lung tissue. They found that testis is also very active. Unfortunately neither group of workers made any studies of the chemical character of the more active steroid formed. The conclusion that estradiol was formed is based solely on the fact that the increased activity approached, but never exceeded, that of an equal amount of this steroid in the assay.

Indirect chemical evidence of the conversion of estrone to estradiol by pregnant rabbit endometrium was obtained by SZEGO and SAMUELS (1943b). Using analytical conditions under which estriol did not give a colour by their procedure, they found that incubation of estrone with this tissue led to a shift of chromogenic material from the phenolic to the non-phenolic fraction. This did not occur with endometrium of pregnant human uterus or bovine non-pregnant uterus. Apparently there are species differences in the metabolism of estrogens in the uterus.

In summary, the estrogens apparently are largely converted into inactive compounds by the liver. They are also excreted in the bile. Other tissues, however, also contain enzymes which will catalyze the interconversion of estrone and estradiol. The inactive excretory products have, so far, not been identified.

C. Metabolism of progesterone

1. *Overall metabolism:* When progesterone is injected into human subjects the amount of pregnane-3α,20α-diol (XXXVI) excreted in the urine is increased

(Fig. 6). This compound is found in the urine of women during the secretory phase of the menstrual cycle and particularly during pregnancy (MARRIAN, 1929), times when the physiological action of the progestins is observed. When progesterone is administered to males or non-pregnant females only 10 to 15 per

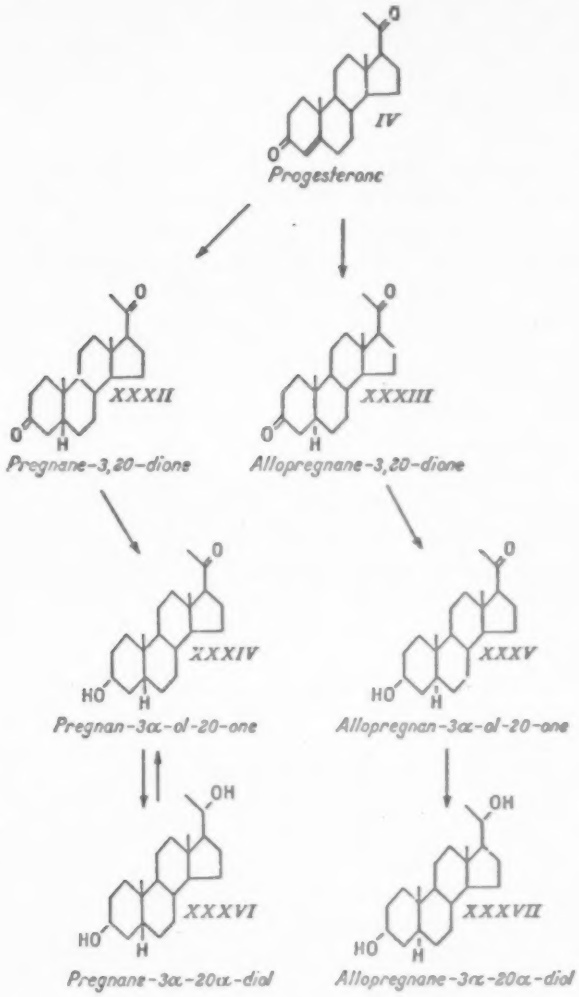


Fig. 6. Probable normal routes of progesterone metabolism.

cent appears as pregnanediol (SOMMERVILLE and MARRIAN, 1950a,b). In pregnancy the recovery is 30 to 50 per cent of the injected hormone. Other steroids which have been found in increased amounts after administration of progesterone are pregnan-3α-ol-20-one (XXXIV), allopregnan-3α-ol-20-one (XXXV) (MARRIAN and GOUGH, 1946), and allopregnane-3α,20α-diol (XXXVII) (KYLE

and MARRIAN, 1951). The greater portion of the injected progesterone remains unaccounted for, however.

The development of an efficient synthesis of 4-C^{14}-progesterone by FUJIMOTO and PRAGER (1953) in which the position of the isotope is definite, has helped in the study of progesterone metabolism. GALLAGHER (1954) administered 4-C^{14}-progesterone to women and found that most of the radioactivity appeared in the urine as neutral steroids conjugated with glucuronic acid. After collection of urine for a total of 3 to 7 days only approximately 30 per cent of the injected activity was accounted for.

RIEGEL *et al.* (1950), using 21-C^{14}-progesterone, followed the metabolism of this steroid in rodents. Within a short time after administration, most of the radioactivity was in the neutral non-saponifiable fraction of the intestinal contents and faeces. A small amount was in the urine and about 10 per cent was in the expired air. Apparently, in the rodent the side chain is split from progesterone to a certain extent. The rapidity of excretion in the rodent, the greater elimination in the faeces, and the presence of C-21 in the expired air have been confirmed by BARRY *et al.* (1952) and GRADY *et al.* (1952). The latter workers used intramuscular injections and found a more prolonged elimination with a smaller proportion appearing as CO_2, but otherwise the results were similar.

2. *Metabolism by specific tissues:* GRADY *et al.* (1952) studied the role of the bile in the metabolism of progesterone in the rodent. They injected radioactive progesterone into rats with bile fistulas and found that all of the radioactive material probably reached the gut by way of the bile duct. If the bile duct were occluded the excretion was almost as rapid, but the urine was now the major pathway. When radioactive material was collected from the bile fistula of a progesterone-injected rat and introduced into the intestinal tract of another rat with a bile fistula the isotope appeared in the bile of this animal. Thus it would seem highly probable that normally some of the metabolites are reabsorbed and re-excreted, a hepatoenteric cycle as originally postulated for the estrogens by CANTAROW *et al.* (1943).

A similar sort of cycle may also exist in the human. ROGERS and MCLELLAN (1951) isolated small amounts of pregnanediol from bile samples of four patients after massive oral doses (500 mg) of progesterone, but the bile extracts showed no progestational activity. WIEST *et al.* (1954) injected 4-C^{14}-progesterone into a woman with a bile fistula but with clinically normal liver function. Within the next 24 hours 30 per cent of the injected radioactivity appeared in the bile, largely as a glucuronoside. The steroids present in largest quantity were reduction products of progesterone; none of the original hormone was found. To what extent these steroids are reabsorbed, metabolized by intestinal bacteria, or excreted in the faeces remains to be determined.

Besides the observations on biliary excretion of the metabolites of isotopic progesterone, other evidence has been obtained that the liver is the principal organ involved in progesterone metabolism. DOSNE (1944), KOCHAKIAN *et al.* (1944), and ENGEL (1946), demonstrated that the hormone implanted in areas

drained by the portal vein was less active than when placed in other regions. In 1949 SAMUELS reported that liver minces would destroy the α,β unsaturated ketone structure as measured by the decrease in ultraviolet absorption at 240 mμ. WISWELL and SAMUELS (1953) studied this reaction in liver homogenates. Oxygen was not essential for the reaction under the conditions used. The reaction was accelerated by metal-binding reagents, indicating that it was easily inhibited by some polyvalent metal. This distinguished it from a citrate-catalyzed system acting on testosterone.

TAYLOR (1954) confirmed these findings. In addition, he demonstrated that DPN would increase the metabolism of progesterone by rat liver slices or homogenates, contrary to the report of Wiswell and Samuels. He isolated allopregnane-3,20-dione (XXXIII) and allopregnane-3α-ol-20-one, indicating that the first step in metabolism was a stericly directed reduction of the double bond, followed by a reduction of the ketone on C-3.

This does not mean, however, that other tissues might not participate in its metabolism. WIEST *et al.* (1954) injected 4-C^{14}-progesterone intravenously into eviscerated rats and extracted urine and tissues at intervals thereafter. Within one hour a considerable amount of the steroid was in more reduced forms. Apparently other tissues have the capability of acting on the progesterone molecule.

As mentioned in the discussion of overall metabolism, pregnane-3α,20α-diol is the most abundant metabolite of progesterone so far isolated from human urine, yet it accounts for less than 20 per cent of the injected dose in normal men and women. GRANT and MARRIAN (1950) investigated the possibility that it, too, might be metabolized. When incubated with liver homogenates under aerobic conditions the steroid disappeared. This reaction required a source of oxygen and was not inhibited by cyanide. It was not, therefore, simply a reversal of the reaction studied by WISWELL and SAMUELS. From such incubations, pregnan-3α-ol-20-one (XXXIV) was isolated (GRANT, 1952). Since this steroid is present in the urine when progesterone is metabolized, the reaction studied by GRANT probably represents a reversal of the last step in the conversion of progesterone to pregnane-3α,20α-diol.

At the present time the mechanisms by which progesterone is metabolized in the animal are very poorly understood. That reductive reactions play a significant role, as with other steroid hormones, seems certain from the urinary studies, but that other types of reactions may be equally important is certainly a possibility.

D. Metabolism of androgens

1. *Overall metabolism:* In 1933 BÜHLER reported that only a small fraction of the biological activity of an androgenic extract injected into a human male could be recovered in the urine of the recipient. Following the identification of testosterone in bull testes extracts both CALLOW (1939) and DORFMAN and HAMILTON (1939) injected testosterone propionate into hypogonadal men and recovered increased amounts of androsterone (V) and etiocholan-3α-ol-17-one

(etiocholanolone) (XLIV; Fig. 7). Like the endogenous steroids, these were present as conjugates with some hydrophilic group and could only be extracted with lipid solvents after acid hydrolysis. After administration of the same

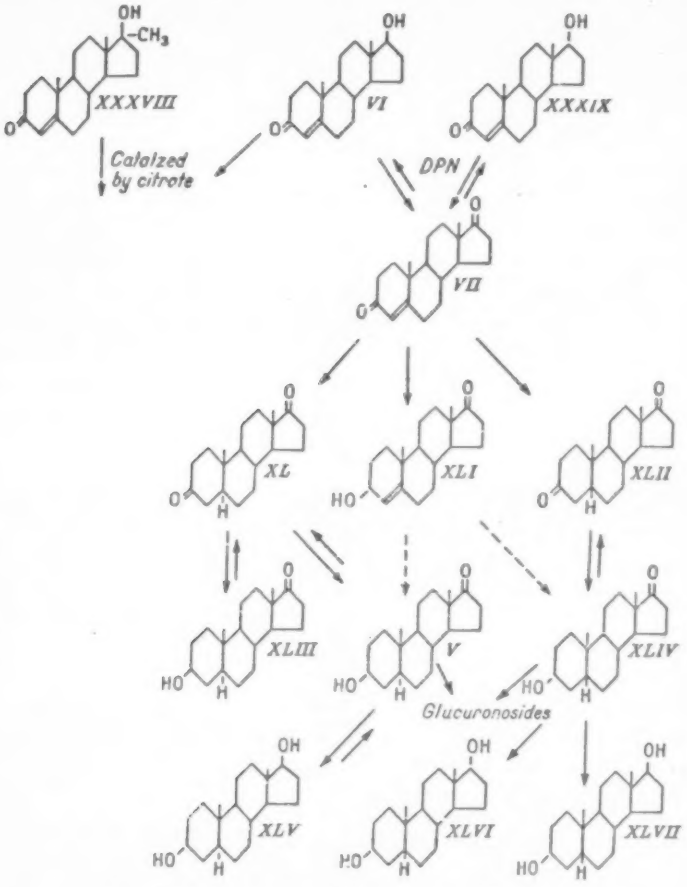


Fig. 7. Probable routes of testis hormone metabolism.
XXXVIII: 17α-methyltestosterone; VI: testosterone; XXXIX: transtestosterone; VII: 4-androsten-3,17-dione; XL: androstane-3,17-dione; XLI: 4-androsten-3α-ol-17-one; XLII: etiocholan-3,17-dione; XLIII: androstan-3β-ol-17-one (epiandrosterone); V: androsterone; XLIV: etiocholan-3α-ol-17-one; XLV: androstane-3α,17β-diol; XLVI: etiocholane-3α,17α-diol; XLVII: etiocholane-3α,17β diol.

hormone to normal women SCHILLER *et al.* (1945) found the same two metabolites and possibly androstane-3α,17β-diol (XLV). Epiandrosterone (androstan-3β-ol-17-one) (XLIII) was also found by DORFMAN (1941) after injection of testosterone into a hypogonadal man.

DOBRINER and LIEBERMAN (1950) administered 90 mg of testosterone daily for 45 days and were able to account for 50 per cent of the injected steroid as

increased output of various compounds. The distribution was: androsterone 24 per cent, etiocholanolone 19 per cent, androstane-3,17-dione (XL) and etiocholane-3,17-dione (XLII) 1 per cent, androstane-3α,17β-diol (XLV) and etiocholane-3α,17β-diol (XLVII) 6 per cent. WEST *et al.* (1951a) injected intravenously 100 to 200 mg testosterone dissolved in serum albumin solution into normal young men and recovered 72 to 76 per cent as excess 17-ketosteroids within 48 hours. Under these conditions no androstan-3β-ol derivatives were found and most of the material was recovered as androsterone and etiocholanolone glucuronides (WEST *et al.*, 1951b). In addition, 3,5-androstadien-17-one was isolated. This could be formed easily from 4-androsten-3α-ol-17-one (XLI) during acid hydrolysis, and would indicate that the latter, a likely intermediate between 4-androstene-3,17-dione and the two saturated 17-keto-3α-ol-steroids, was probably excreted.

Considerable work has been done with isotopic testosterone of various types. In most particulars it has agreed with the chemical studies. GALLAGHER *et al.* (1951) injected 100 mg deuterium-labelled testosterone into a man and collected the urine for three days thereafter. Deuterium was found in androstane-3,17-dione, etiocholane-3,17-dione, 2-androsten-17-one, androsterone and etiocholanolone. The 2-androsten-17-one was an artifact formed from androsterone during the acid hydrolysis. Twenty-six per cent of the isotopic hormone was accounted for as androsterone and 10 per cent as etiocholanolone. No excess isotope was found in the β-hydroxy (epiandrostane) fraction. After intravenous infusion of 4-C^{14}-testosterone into a man, 51 per cent of the radioactivity was recovered in the urine during the first day (GALLAGHER, 1954). When urine was collected for three days after a similar injection into a woman 77 per cent of the radioactivity was recovered, 90 per cent of which was as steroid glucuronides. Again most of the isotope was in androsterone and etiocholanolone. It seems quite clear, therefore, that the major end products of testosterone metabolism are these two compounds, which are excreted as the glucuronides.

The overall method has also been used to determine probable intermediate steps in testosterone metabolism. Since 4-androstene-3,17-dione would appear to be a possible key intermediate in the conversion, DORFMAN and HAMILTON (1940) administered this compound to a hypogonadal man and found increased amounts of both androsterone and etiocholanolone. GALLAGHER and coworkers (1951) injected deuterium-labelled 4-androstene-3,17-dione and recovered 43 per cent as androsterone and etiocholanolone. It could, therefore, be an intermediate in testosterone metabolism.

DORFMAN and HAMILTON (1940) administered androstane-3,17-dione and androstane-3α,17β-diol as possible intermediates. Only androsterone, and not etiocholanolone, was recovered. The same was true when deutero-androstanedione was given (GALLAGHER *et al.*, 1951). It would appear, therefore, that once the double bond is reduced, no interconversion of the isomers can take place. Apparently the reduction of the double bond is not sterically specific and accounts for the two isomeric end products.

Since in no case has all of the administered steroid been accounted for, and

since the diols which would result from reduction of androsterone and etiocholanolone have been found after testosterone administration, it is obvious that the two major end products can be further metabolized. Probably the two 17-ketosteroids are found in largest quantity in the urine because they are conjugated rapidly and thus eliminated from the degradative systems.

While the glucuronides are important conjugates of the steroids, the sulphate radical is also used. VENNING *et al.* (1942) isolated androsterone sulphate from the urine of a man with an interstitial cell tumour of the testis. MUNSON *et al.* (1944) isolated dehydroepiandrosterone sulphate from the urine of normal young men. According to LIEBERMAN *et al.* (1954) the 3β-hydroxy-17-ketosteroids are all excreted as sulphates while the 3α-hydroxy-17-ketosteroids are generally conjugated as glucuronidates.

The overall studies in human beings, therefore, indicate that testosterone can be converted to its fellow hormone, 4-androstene-3,17-dione after it is secreted, and that this compound is then reduced stepwise to androsterone and etiocholanolone which conjugate readily with glucuronic acid and are largely excreted as the glucuronidates. The work with isotopes would indicate that the reduction at the double bond is not stericly specific but that of the 3-ketone is.

Soon after the urine was recognized as a major route of excretion of steroid hormones in men, attempts were made to use laboratory animals for excretion studies. It was very quickly recognized that there are marked species differences. Most domestic animals do not excrete significant amounts of the steroids found in human urine. The guinea pig and the rabbit excrete small amounts normally and show significant increases when steroid hormones are administered. The equine, as already noted, is unique in that it exceeds the human in number and amounts of steroids in the urine. Even the stallion is an excellent producer of estrogens. These species differences have been emphasized by the studies with isotopic steroids.

BARRY *et al.* (1952) administered 4-C^{14}-testosterone intraperitoneally to rats and mice. As in the studies of WEST *et al.* (1951) on intravenously injected testosterone in human beings, the hormone was rapidly cleared from the circulation. The greater part appeared in the bile and was excreted in the faeces. While no isolations were carried out, the material in both faeces and urine did not resemble either the conjugated or free forms of the metabolites of testosterone as found in human urine. The radioactivity was not extractable with ether after strong acid hydrolysis but was extracted with n-butanol. It could not be washed from this solution by 5 per cent sodium hydroxide and therefore was not present as a conjugated acid. Obviously the metabolism of testosterone differs markedly in the rodent from that in man. Generalizations must not be made from one species to others without supporting evidence from all.

2. *Metabolism by specific tissues:* BISKIND and MARK (1939) first demonstrated the role of the liver in the metabolism of testosterone. They implanted pellets of testosterone in the spleens of immature and castrated rats. As long as the blood from the spleen flowed only to the liver through the portal vein there was no

androgenic action. When, however, the spleens were transplanted into the axilla or developed venous connections with the body wall, the rats immediately showed androgenic changes similar to those seen when the pellets were implanted under the skin. DANBY (1940) first obtained destruction of testosterone by the isolated perfused liver. CLARK and KOCHAKIAN (1944, 1947) incubated the hormone under aerobic conditions with rabbit liver slices. Besides unchanged testosterone they were able to isolate 4-androstene-3,17-dione and a small amount of epitestosterone (4-androstene- 17α-ol-3-one) (XXXIX). Recently KOCHAKIAN *et al.* (1952a) reported similar conversions with homogenates of rabbit liver and kidney. The system seems reversible since KOCHAKIAN *et al.* (1952b) were able to obtain 4-androstene-3,17-dione from incubation of rabbit liver or kidney homogenates with epitestosterone. Niacin added to the homogenate increased the conversion to the dione, indicating that DPN is probably the hydrogen acceptor (KOCHAKIAN and NALL, 1953).

SAMUELS *et al.* (1947) used the absorption band at 240 mμ characteristic of the α,β unsaturated ketones to measure changes in the conjugated system in ring A of testosterone, and the Zimmermann reaction to measure the formation of 17-ketones. Tissue minces or homogenates were incubated with 1 or 2 μmols of the steroid in Krebs phosphate buffer, extracted with ether or other lipid solvent, and chromatographed on an aluminium oxide column. This gave steroid fractions sufficiently pure for the determinations indicated. Of a large number of rat tissues tested, only the liver and kidney were found to act on the hormone. Kidney tissue was much less active than the liver (WEST and SAMUELS, 1951).

When liver slices or minces were used, there was first a decrease in the α,β unsaturated system and appearance of the 17-ketone group. The latter then went through a maximum and decreased. After long incubation both groups had disappeared (SAMUELS, 1949).

When homogenates were used, there was very little effect until either diphosphopyridine nucleotide (DPN) or citrate was added (SWEAT and SAMUELS, 1948). The action of the two cofactors was not the same. The 17-ketosteroids markedly increased when DPN was added, sometimes exceeding the disappearance of the α,β unsaturated system. When citrate was used, however, there was no appearance of 17-ketones. If both were added, the effect on the α,β unsaturated system was approximately additive while the appearance of 17-ketones was intermediate. Other members of the Krebs cycle which were tested would not take the place of citrate.

The obligatory nature of the 17-oxidation in the DPN sequence and its unimportance for the citrate reaction was shown by LEVEDAHL and SAMUELS (1950). When 17α-methyltestosterone (XXXVIII) was used as substrate, thus blocking oxidation at C-17, DPN had no effect on the rate at which the α,β unsaturated ketone disappeared but citrate was still effective.

SWEAT *et al.* (1950) demonstrated that the DPN-activated enzyme was in the supernatant after high speed centrifugation. They fractionated the proteins with ammonium sulphate and found that the fraction in solution at half

saturation but precipitated by full saturation catalyzed only one reaction, the oxidation of carbon-17 with the reduction of DPN. 4-Androstene-3,17-dione and unchanged testosterone accounted for all the original substrate. Thus the sequence in this system seems to be first the oxidation of the 17β-hydroxyl to a ketone, and then in the crude homogenate the reduction of the double bond, and ultimately, reduction of both ketones. The citrate-catalyzed system acts directly on the conjugated unsaturated structure in ring A without preliminary oxidation at position 17.

In their original studies, SAMUELS *et al.* (1947) did not find that rat prostate had any effect on testosterone. Recently WOTIS and LEMON (1954), using human prostatic tissue, have reported that this also causes the disappearance of the ultraviolet absorption due to the α,β unsaturated ketone system. The activity is much less than that of the liver, however. Whether this reaction has anything to do with the hormonal action of testosterone remains to be determined.

The later steps in the metabolism of the androgens have been investigated by SCHNEIDER and MASON and by KOCHAKIAN. SCHNEIDER and MASON (1948b) incubated androsterone with rabbit liver slices. They obtained androstane-3α, 17β-diol as the main product, with small amounts of androstane-3,17-dione and epiandrosterone (XLIII). From etiocholan-3α-ol-17-one they obtained etiocholane-3α,17α-diol (XLVI), etiocholane-3α,17β-diol, and etiocholane-3,17-dione. KOCHAKIAN and APOSHIAN (1952) incubated the product androstane-3α,17β-diol with liver and kidney homogenates from rats, rabbits and guinea pigs. They isolated small amounts of androsterone, epiandrosterone and androstane-3,17-dione. This series of reactions, therefore, seems reversible.

E. Metabolism of adrenal steroid hormones

1. *Overall metabolism:* Several steroids in the urine have been considered probable metabolites of adrenal hormones because of the presence of oxygen at carbon-11. Others like dehydroepiandrosterone are present in the urine of castrate men and women and increased in the presence of adrenal hyperplasia and adrenal tumors. It was only with the availability of large supplies of cortisone in 1949, and of 17-hydroxycorticosterone in 1951, however, that the metabolism of the adrenal hormones could be studied adequately. It was soon clear that the strong acid hydrolysis usually used in isolation of steroids from urine would destroy the dihydroxyacetone side chain. If, instead, urine to which such compounds had been added was adjusted to *p*H 1 and allowed to stand overnight at room temperature little loss occurred. Urine so treated yielded larger amounts of compounds with the α-ketol side chain than urine extracted immediately without adjustment of *p*H. The amounts recovered by this procedure, however, were very small and showed only a rough correlation with the clinical condition of the patient.

Not only were the amounts of steroids with the α-ketol side chain very small when normal urine was extracted at *p*H 1, but the yields after administration of the cortical hormones were very low. When DAUGHADAY *et al.* (1951) compared

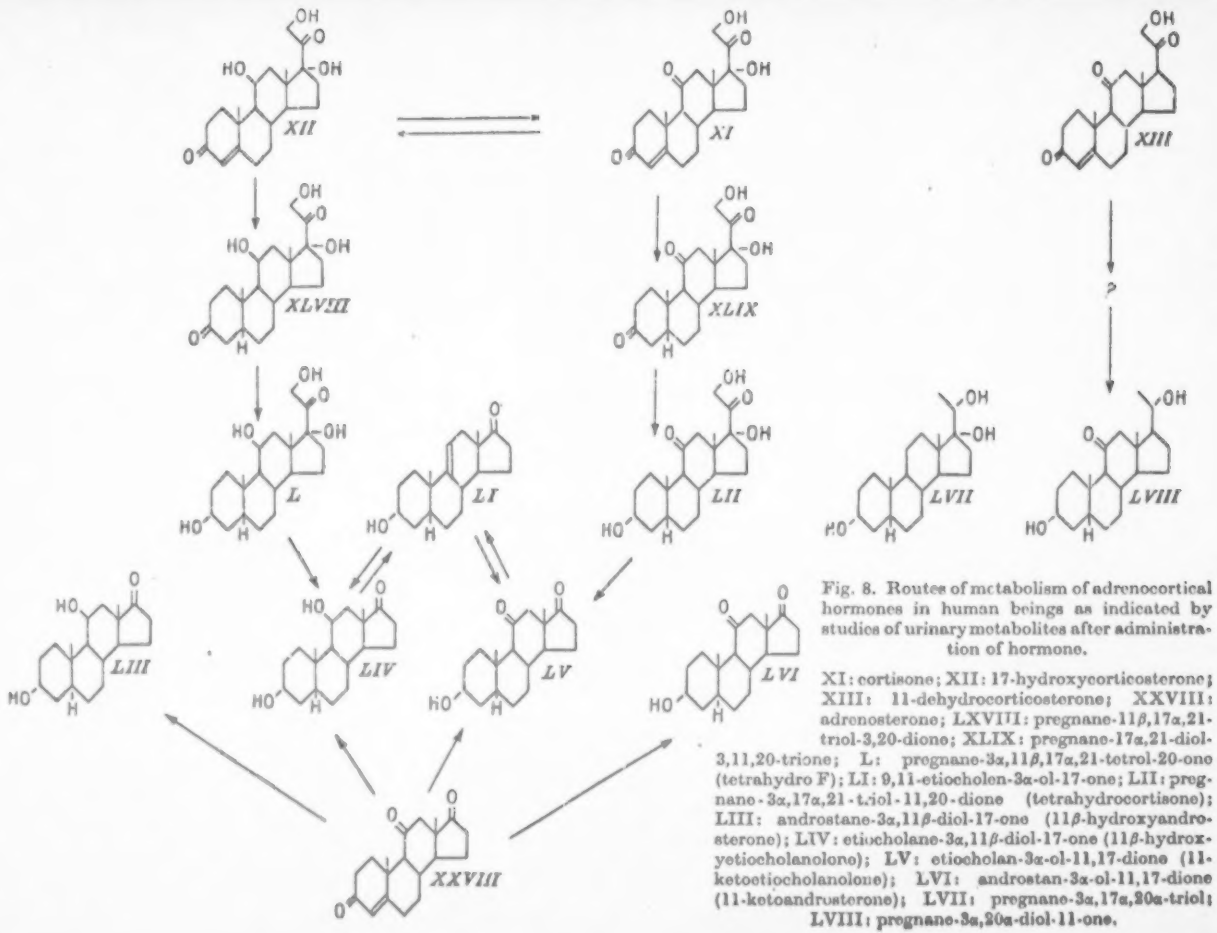


Fig. 8. Routes of metabolism of adrenocortical hormones in human beings as indicated by studies of urinary metabolites after administration of hormone.

XI: cortisone; XII: 17-hydroxycorticosterone; XIII: 11-dehydrocorticosterone; XXVIII: adrenosterone; LXVIII: pregnane-11β,17α,21-triol-3,20-dione; XLIX: pregnane-17α,21-diol-3,11,20-trione; L: pregnane-3α,11β,17α,21-tetrol-20-one (tetrahydro F); LI: 9,11-etiocholen-3α-ol-17-one; LII: pregnane-3α,17α,21-triol-11,20-dione (tetrahydrocortisone); LIII: androstane-3α,11β-diol-17-one (11β-hydroxyandrosterone); LIV: etiocholane-3α,11β-diol-17-one (11β-hydroxyetiocholanolone); LV: etiocholan-3α-ol-11,17-dione (11-ketoetiocholanolone); LVI: androstan-3α-ol-11,17-dione (11-ketoandrosterone); LVII: pregnane-3α,17α,20α-triol; LVIII: pregnane-3α,20α-diol-11-one.

the yields by this procedure with those after hydrolysis with β-glucuronidase, the latter were more than ten times as great. Cox and Marrian (1953a) have also studied the types of conjugates present. There are small amounts of chloroform-soluble conjugates which are not split by β-glucuronidase, but the major portion of the compounds with α-ketol side chains are non-chloroform-soluble glucuronides.

The development of a paper chromatographic technique for the separation of polar steroids enabled Zaffaroni *et al.* (1950) to demonstrate the presence of cortisone (XI), 17-hydroxycorticosterone (XII) and at least two more polar compounds in urine extracts. Lieberman *et al.* (1950a) identified pregnane-3α,17α,21-triol-11,20-dione (LII) (tetrahydrocortisone) (Fig. 8). Schneider (1952a) also isolated pregnane-17α,21-diol-3,11,20-trione (XLIX) (dihydrocortisone). After administration of either cortisone or 17-hydroxycorticosterone all of these compounds plus 11-ketoetiocholanolone (LV), 11β-hydroxyetiocholanolone (LIV) (Lieberman *et al.* 1950b, 1951; Mason, 1950) and the dehydration product, 9,11-etiocholen-3α-ol-17-one (LI) (Burstein *et al.* 1953a,b) were increased. After oral administration of 17-hydroxycorticosterone the latter workers also isolated the product of reduction in ring A, pregnane-3α,11β,17α,21-tetrol-20-one (L). Lieberman *et al.* (1951) reported that 11β-hydroxyandrosterone (LIII) was increased after cortisone but Burstein *et al.* (1953a) were unable to confirm this. The major product is largely tetrahydrocortisone (LII), even after administration of 17-hydroxycorticosterone (Burstein *et al.* 1953b). It appears, therefore, that this is the major excretion product and that it conjugates readily with glucuronic acid. After administration of cortisone or 17-hydroxycorticosterone the total yield of compounds having the dihydroxyacetone side chain is 25 to 35 per cent of the dose (Tyler *et al.* 1953).

Another reaction was indicated by the studies of Mason (1948). When 11-dehydrocorticosterone (XIII) was administered to two patients with *Addison's* disease, pregnane-3α,20-diol-11-one (LVIII) was isolated from the urine. This would involve reduction of the hydroxyl group on carbon-21 to a methyl group as well as the reduction of the 20-ketone to a hydroxyl. Marrian and co-workers (Marrian, 1951; Cox, 1952) have shown that normal urine of both sexes contains steroidal substances which yield acetaldehyde on oxidation with periodate. This would indicate that there are compounds present which have a hydroxyl on carbon-17 and an oxygen function on carbon-20 but a methyl group on carbon-21. Cox and Marrian (1953b) were able to isolate pregnane-3α,17α,20α-triol (LVII) as the major component.

When 500 mg per day of either cortisone or 17-hydroxycorticosterone was administered by mouth to normal young men for five days no etiocholanolone or androsterone could be isolated from the urine (Burstein *et al.* 1953a, 1953b). Since these metabolites of the testicular hormones were present in normal amounts before the cortical hormones were administered, this suggests that either the production of hormones by the testis was depressed or that there was competition for some of the enzymes involved in their metabolism.

The isotope technique has not been used extensively in the study of the metabolism of the adrenal steroids because of the unavailability of proper labelled compounds. FUKUSHIMA *et al.* (1951) labelled 17α-hydroxyprogesterone (XXIII) with deuterium by a catalytic exchange process. When this was administered some of the isotope appeared in the 17-ketosteroids, indicating that the side chain was split from a portion of the molecules. Since such a reaction occurs in the testis and probably the adrenal cortex, this may have simply been due to penetration of the administered steroid into these glands. GALLAGHER (1954) has used cortisone labelled with tritium. Approximately 79 per cent of the isotope was at carbon-16 where it would be unstable if the side chain were split off, 20 per cent was at carbon-4 and 5 per cent at carbon-21. When this material was given to humans, 50 to 90 per cent could be accounted for in the urine, largely as glucuronides. The side chain, therefore, was not split off from the greater portion.

In addition to the overall changes in the metabolically active hormones of the adrenal cortex, the metabolism of the 17-ketosteroids and the electrolyte hormone secreted by the adrenals must be considered. Although the adrenal 17-ketosteroids have not been clearly identified, GASSNER *et al.* (1951) obtained suggestive evidence of the presence of 11β-hydroxy-4-androstene-3,17-dione (VIII) and adrenosterone (4-androstene-3,11,17-trione) (XXVIII) in bovine adrenal venous blood. SAVARD *et al.* (1953) administered 100 mg of adrenosterone per day for 10 days to a 61-year-old man and isolated the products thereof from the urine. The principal product was 11β-hydroxyandrosterone (LIII). In addition, increased quantities of 11-ketoandrosterone (LVI), etiocholane-3α,11β-diol-17-one (LIV) and etiocholan-3α-ol-11,17-dione (LV) were found. As in testosterone metabolism, the reduction of the double bond in ring A does not seem to be sterically specific, but the alcohols resulting from reductions of the ketones are solely 3α and 11β.

Since the structure of the electrolyte regulating hormone, aldosterone, has been determined so recently, very little can be said about its metabolism. One significant observation has been made by LUETSCHER *et al.* (1953). Persons with oedema excrete increased amounts of a steroid (or steroids) having the same biological and chromatographic characteristics as this compound.

From the overall studies described the following types of reactions are indicated: (1) reduction of the double bond in ring A, apparently sterically directed toward the pregnane (H at C-5 *cis*) configuration, (2) reduction of the 3-ketone to a 3α-ol, (3) reduction of the hydroxyl at carbon-21 to a methyl group, (4) reduction of the 20-ketone to a 20α-ol, and (5) splitting off of the side chain with the formation of 17-ketones. In addition, it would appear that the system $-OH \rightleftarrows = O$ on carbon-11 is reversible. Thus far there is no evidence, however, that the oxygen, once introduced in this position, can be eliminated.

2. *Metabolism in specific tissues:* BUTT *et al.* (1951a) and FAJANS *et al.* (1953) observed that the increase in 17-ketosteroids which normally follows the administration of cortisone and 17-hydroxycorticosterone was reduced in hepatic

cirrhosis. BROWN *et al.* (1954) have found that the removal of intravenously infused 17-hydroxycorticosterone is proportionate to concentration in both normal and cirrhotic subjects but that the rate of removal is definitely slower in the latter group. Levels of endogenous 17-hydroxycorticosteroids in the plasma of persons with hepatic disease are normal, but the ratio of conjugated to free steroids in the urine is markedly reduced (12 : 1 to 20 : 1 compared with normal ratios of 24 : 1 to 60 : 1), indicating interference with conjugation.

The splenic implantation technique has been used to study the action of the liver on active adrenocortical steroids. BURRILL and GREENE in 1942 implanted single pellets of desoxycorticosterone acetate in the mesenteries of adrenalectomized rats. Those with small pellets died almost as soon as the untreated controls, although a group with subcutaneous pellets of the same size lived and gained weight. If pellets three times as large were implanted mesenterically, however, the adrenalectomized rats survived longer. It appeared, therefore, that this steroid could be inactivated by the liver but that the capacity of the metabolic system was limited.

NELSON *et al.* (1951) compared the levels of 17-hydroxycorticosteroids (those substances which have the dihydroxyacetone side chain) in femoral arterial and venous blood of humans. There was no measurable removal by the leg tissues even when severely inflamed. The investigators did not determine, however, whether ring A was affected. NELSON and HARDING (1952) also compared arterial and venous levels across various organs in the dog. Only in the case of the liver were significant differences found. The concentrations in the hepatic vein were always lower than in the arterial blood, but those in the portal vein were higher. This has been confirmed by EIK-NES (1953).

HECHTER and co-workers have used the perfusion technique to study the metabolism of the cortical homones by the liver. They find that cortisone, 17-hydroxycorticosterone, 11-desoxycorticosterone (IX) and 17α-hydroxy-11-desoxycorticosterone (XXIV) all yield a variety of metabolites in a basically similar pattern. A series of chromatographic systems on paper was used to distinguish the products. On perfusion of cortisone they obtained 14 spots which gave reactions for the α-ketol side chain, 3 which gave the 17-ketosteroid reaction and 8 others which reacted with dinitrophenylhydrazine but did not have the α-ketol side chain. Other products which would not react with any of the colour reagents used might have been present (HECHTER *et al.* 1953a; CASPI *et al.*, 1953). The major products were allopregnane-3α,11β,17α,21-tetrol-20-one (LXIII), allopregnane-3α,17α,21-triol-11,20-dione (LXIV), and allopregnane-3β,17α,21-triol-11,20-dione (LXV) (Fig. 9). Other products isolated and identified were allopregnane-3β,11β,17α,21-tetrol-20-one (LXII), 17-hydroxycorticosterone (4-pregnene-11β,17α,21-triol,3,20-dione), 4-pregnene-17α,20β,21-triol-3,11-dione (LX) and adrenosterone (XXVIII). There was also some evidence that the two dihydro derivatives of cortisone (double bond reduced) were present. AXELROD and MILLER (1953), using perfusion of rat liver, confirm the hydroxylation at C-11 and C-17, the formation of 17-ketosteroids, reduction in ring A to yield both the 3α- and 3β-hydroxy compounds,

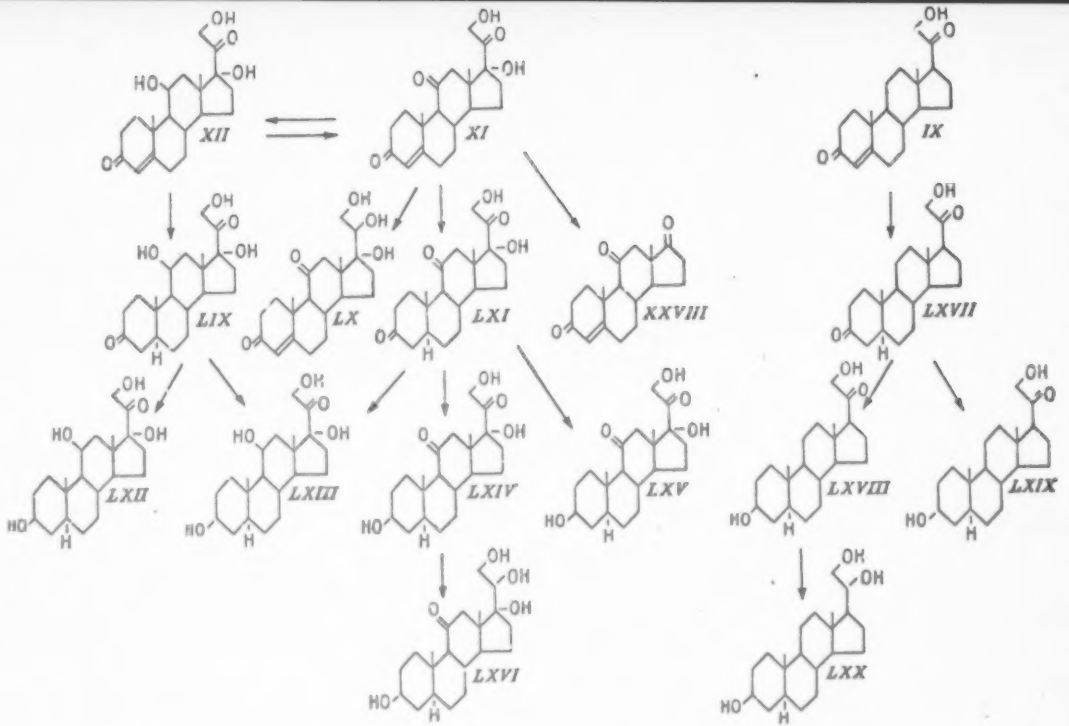


Fig. 9. Metabolic routes of adrenocortical hormones as indicated by *in vitro* studies with liver tissue of animals. No data from incubations with human tissue.

IX: 11-desoxycorticosterone; XI: cortisone; XII: 17-hydroxycorticosterone; XXVIII: adrenosterone; LIX: allopregnane-11β,17α,21-triol-3,20-dione; LX: 4-pregnene-17α,20β, 21-triol-3,11-dione; LXI: allopregnane-17α,21-diol-3,11,20-trione; LXII: allopregnane-3β,11β,17α,21-tetrol-20-one; LXIII: allopregnane-3α,11β,17α21-tetrol-20-one; LXIV: allopregnane-3α,17α,21-triol-11,20-dione; LXV: allopregnane-3β,17α,21-triol-11,20-dione; LXVI: allopregnane-3α,17α,20α,21-tetrol-11-one; LXVII: allopregnan-21-ol-3,20-dione; LXVIII: allopregnane-3β,21-diol-20-one; LXIX: allopregnane-3α,21-diol-20-one; LXX: allopregnane-3β,20α,21-triol.

and reduction of the ketone group on C-11 to hydroxyl. They did not find the latter reaction reversible since, while cortisone yielded 17-hydroxycorticosterone, incubation of the latter did not yield cortisone.

The conversion of corticosterone to 17-hydroxycorticosterone by liver homogenates has been reported by FISH *et al.* (1953). Homogenates of kidney, heart muscle, and ovary were ineffective.

SCHNEIDER and HORSTMANN (1951, 1952) have studied the metabolism of cortical steroids by tissue slices. Liver was by far the most active tissue, kidney was about 1/3 as active and adrenal homogenate showed slight but measurable activity, based on disappearance of the α,β unsaturated ketonic system. The same order was found when loss of reductive character due to the α-ketol side chain was the criterion. A number of other tissues was inactive. From incubation of desoxycorticosterone four metabolites were identified, all members of the allopregnane system. They were allopregnane-3β,21-diol-20-one (LXVIII), allopregnane-3α,21-diol-20-one (LXIX), allopregnane-21-ol-3,20-dione (LXVII) and allopregnane-3β,20α,21-triol (LXX) (SCHNEIDER, 1952b). Again both isomeric alcohols due to the reduction of the 3-ketone were found while only the allo configuration at C-5 was identified. Similar derivatives of cortisone were isolated after its incubation. Either further metabolism of these hepatic metabolites takes place in other tissues or the isomerisms on C-3 and C-5 represent a species difference. The overall studies which yielded only 3α-ols and pregnane derivatives were done in human subjects while tissues from other mammals were used in the *in vitro* studies.

Obviously a series of enzymes must be involved in the transformations. So far, however, only one system has been studied in any detail. AMELUNG *et al.* (1953a,b) have demonstrated the reduction of 11-ketosteroids to 11β-hydroxysteroids with microsomes from liver homogenates. DPN, ascorbic acid, citric acid and oxygen stimulated the conversion; As_2O_3, NaCN and nitrogen inhibited it.

While he has not isolated the system involved, SCHNEIDER (1953) has shown that the reduction of cortisone follows first order kinetics. The introduction of tetrahydrocortisone (pregnane-3α,17α,21-triol-11,20-dione) will partially inhibit the reduction, probably by mass action. It will also act as competitive inhibitor of cortisone in the glycogen deposition test.

The dominant role of the liver in the metabolism of the cortical steroids is apparently important in the changes in concentration of these steroids during acute stresses. TYLER *et al.* (1953) have shown that the increase in concentration of 17-hydroxycorticosteroids which occurs after heavy surgery is often more rapid and greater than can be produced by maximal doses of adrenocorticotropic hormone. These increases are associated with decreased bromsulphthalein clearance, a measure of hepatic function. EIK-NES (1953) measured the output of 17-hydroxycorticosteroids from one adrenal of dogs before and after administration of febrile doses of a bacterial pyrogen. There was a transient increase which returned to normal within 45 minutes. The peripheral concentrations were maintained at high levels for a much longer period. He

then studied the effect of the pyrogenic dose on the levels of these steroids in the arterial, portal and peripheral blood. All increased, but the hepatic venous levels, which were originally lowest, rose most, the arterial next and the portal venous concentration least, so that they tended to approach a common value. This would be expected if removal of the 17-hydroxycorticosteroids, and consequently their reabsorption, were reduced. Since the adrenal steroids are anti-inflammatory and antitoxic, this would seem to be an auxiliary way of making higher concentrations available to the tissues in time of need.

The results of all types of experiment seem to agree that the major catabolic reactions are reductive and that the products are increased in hydrophilic character by conjugation with glucuronic acid. An important exception is the splitting of the side chain from 17-hydroxy-20-ketosteroids. Apparently in lower animals, and perhaps in the human, the bile plays an important but unexplained role since large amounts seem to be excreted into the gut but reabsorbed later.

F. The problem of dehydroepiandrosterone

In 1934 BUTENANDT and DANNENBAUM isolated dehydroepiandrosterone from human urine. Along with it they also obtained 5-androsten-3-chloro-17-one, later shown to be an artifact produced from dehydroepiandrosterone during the hydrolysis of the conjugates with strong hydrochloric acid. Dehydroepiandrosterone and its reduction product epiandrosterone are the only C_{19} compounds in the urine which have the hydroxyl group at C-3 in the *cis* or β position similar to that in cholesterol. Like cholesterol, they yield insoluble digitonides, and this has been used as a means of determining them in urine extracts. Earlier observations indicated that 5 to 15 per cent of the ketosteroids in normal urine were of this type. In adrenal hyperplasia dehydroepiandrosterone is increased and in some adrenal carcinomas makes up the greater part of the very high excretion of 17-ketosteroids.

While DORFMAN and FISH (1940) isolated epiandrosterone in increased quantities from the urine of a man given testosterone, the work of GALLAGHER *et al.* (1951) and GALLAGHER (1954) with isotopes indicates that no significant amount of β-ketosteroids arises from this compound. It has been assumed that all of the β-ketosteroids probably arise from the adrenal cortex, either directly or as metabolites of adrenal steroids. As already pointed out, however, the known hormones of the adrenal cortex, cortisone, 17-hydroxycorticosterone and corticosterone, do not yield dehydroepiandrosterone as a major urinary metabolite. The same is true for 11-desoxycorticosterone.

Since dehydroepiandrosterone is so easily converted into other substances during boiling with strong acid, other methods of hydrolysis have been tried. Since its conjugation appears to be with sulphates (MUNSON *et al.*, 1944), bacterial sulphatases have been tried but without great success. Using a system of continuous extraction during mild acid hydrolysis introduced by LIEBERMAN and DOBRINER (1948), LIEBERMAN *et al.* (1954) found that the amount of β-ketosteroids, largely dehydroepiandrosterone, then represented 30 to 50 per

cent of the total 17-ketosteroids. The total yield of 17-ketosteroids was also increased, indicating that the degradation of the β-ketosteroids in the standard acid hydrolysis had been sufficiently extensive to destroy their ability to react with the Zimmermann reagent. COHEN and ONESON (1953), using dioxane-trichloracetic acid hydrolysis, also found that the proportion of β-ketosteroids in urine was much higher than heretofore assumed. MIGEON and PLAGER (1954) have found that dehydroepiandrosterone is the major 17-ketosteroid in human blood, present as a conjugate similar to, if not identical with, that in the urine. Where does this steroid arise?

The products of the degradation of both the C_{19} and C_{21} steroids with the α,β unsaturated ketone structure in ring A appear, in humans, always to be the 3α-hydroxy derivatives. It seems, therefore, that dehydroepiandrosterone arises from the metabolism of some other type of steroid. It has been noted that 5-pregnen-3β-ol-20-one plays a key role in the synthesis of the non-benzenoid hormones. If it were possible for this compound to undergo 17-hydroxylation and the 5-pregnene-3β,17α-diol-20-one (XX) so formed was acted upon by the side chain-splitting enzyme known to be present in the testis and probably present in the adrenal cortex, dehydroepiandrosterone (XXII) would result (LIEBERMAN and TEICH, 1953). If this hypothesis proves true, this steroid must be looked upon as a hormone (see Fig. 4).

This compound, as in the case of other steroids found as conjugates in the urine, can undergo further metabolism. MASON and KEPLER (1947) injected the acetate of dehydroepiandrosterone intramuscularly into patients who, due to adrenal deficiency, had no endogenous production. The major metabolites excreted were androsterone and etiocholanolone. Since the hydroxyl group on carbon-3 in these compounds is *trans* instead of *cis*, it is highly probable that the first step in this sequence of reactions was oxidation to 4-androstene-3,17-dione. From this point the catabolic reactions would be similar to those of testosterone. Some reactions on ring D apparently took place, however, without preliminary oxidation of ring A since they isolated small amounts of 5-androstene-3β,17β-diol (LXXI) and 5-androstene-3β,16α,17β-triol (LXXII) (Fig. 10). The former would be a reduction of the ketone group similar to the conversion of androsterone to androstane-3α,17β-diol while the latter would be the result of addition of water to the 16-enol form. SCHNEIDER and MASON (1948a) incubated dehydroepiandrosterone with rabbit liver slices under aerobic conditions and isolated both 5-androstene-3β,17β-diol and 5-androstene-3β,16α,17β-triol. Probably, as in the case of androsterone and etiocholanolone, the reason that dehydroepiandrosterone appears in relatively large amounts in the urine is the ease with which it is enzymically conjugated. In this case the preferred acid group appears to be sulphate.

G. General Summary

Like most important functions in the living organism the maintenance of the steroid hormone environment is a complex and dynamic process. The reactions bear little resemblance to the synthetic reactions of classical organic chemistry.

This is because energy barriers are bypassed by a series of enzymes specific for steroids.

The non-benzenoid steroid hormones, like cholesterol, can be built up from two carbon fragments. Every tissue forming these compounds appears to be able to form them from simple units. Cholesterol itself can be converted into adrenal hormones by the adrenal glands but there is no conclusive evidence

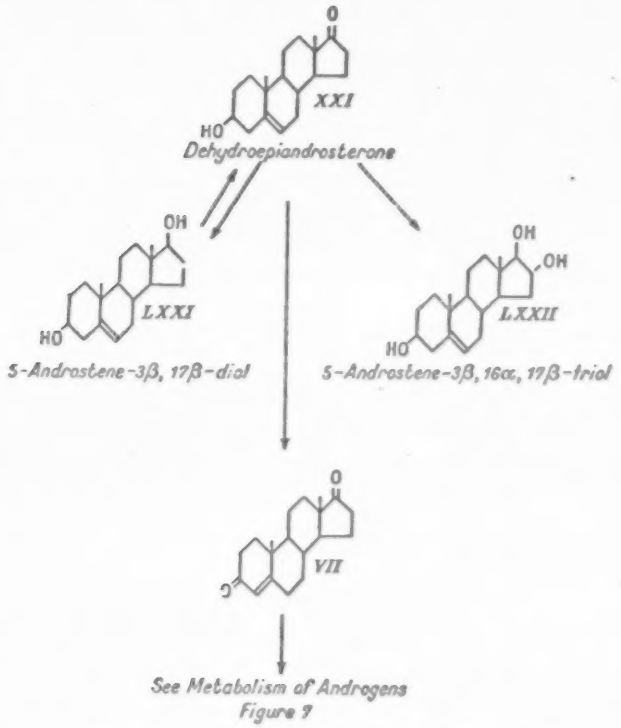


Fig. 10. Metabolic products of dehydroepiandrosterone.

that this step is essential in steroid hormone synthesis. The work of HEARD indicates that cholesterol is not a precursor of the benzenoid steroid hormones. Contrary to the supposition that they simply represent a further step in dehydrogenation of the basic steroid nucleus, it seems that the benzenoid rings may not even arise from two carbon fragments, but have an origin entirely different from that of the saturated portion of the molecule. The isotope studies indicate that even equilin and equilenin are not simply steps in the further dehydrogenation of estrone, but originate independently.

The first precursor which can be linked clearly to the steroid hormones is 5-pregnen-3β-ol-20-one. The steps by which this compound can be converted into any one of the hormones have been demonstrated in isolated tissues as

discussed in the section on biosynthesis. The indications are that the differences between the enzyme systems of the placenta, corpus luteum, testis and adrenal cortex are quantitative rather than qualitative, and that specialization among these cells, which all arise from the region of the genital ridge, consists in development of the relative dominance of certain enzyme systems.

The oxidation of the steroids at position 11 is a good example of the ease with which enzymes can carry out reactions which are very difficult by ordinary synthetic methods. Using non-enzymic methods, the introduction of oxygen in this position into such compounds as 11-desoxycorticosterone or 11-desoxy-17-hydroxycorticosterone is an involved process which requires the production of a double bond in a stable position of the steroid nucleus and subsequent specific hydration without overall disturbance of the more reactive groups. Yet this reaction occurs rapidly and quantitatively at 37°C in the presence of adrenal mitochondria and certain cofactors. In steroid metabolism the limiting factor is not the difficulty of the steroid reaction but the stability of the enzymes involved.

Steric position of the alcohol group in ring A seems to determine the availability of a steroid as a precursor of the hormones which contain the α,β unsaturated ketone structure in this ring. Only those with the 3β (*cis*) configuration will react with the dehydrogenases in the synthesizing tissues. On the other hand, the reducing enzymes of the liver form largely the 3α-hydroxy compounds. Thus there is little re-utilization. As a consequence, the factor which determines the output of any steroid hormone is the immediate concentration of the pituitary or placental hormone which stimulates its production.

The liver appears to be the major organ involved in the inactivating reactions. These reactions, with the exception of the splitting of the side chain and the oxidation of an alcohol group on carbon-17 to a ketone, appear to be reductive in nature. The steroids can be reduced as far as the saturated polyalcohols, perhaps farther. The major products found in the urine, however, are not carried to this state of reduction, probably because intermediates such as androsterone or tetrahydrocortisone are readily conjugated with such hydrophilic groups as glucuronic or sulphuric acid, and are thus filtered out.

The ease of conjugation relative to the rapidity of further reduction reactions thus determines the form of the excretory products. As yet, no one has accounted for all of an administered steroid, even when isotope is used to identify amounts approaching the physiological range. Other reactions may form compounds which, while not important quantitatively, have significant physiological action. And no one has yet identified the form in which the hormone acts in the target tissue. Many problems still remain. But the work thus far indicates that many reactions involving steroids take place in the animal body, and that the turnover of any of these physiologically active compounds is very large in comparison with their concentration. The importance of the endocrine system in the adjustment of the organism to environment probably depends on this dynamism.

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